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INTERGENERATIONAL EFFECTS OF NICOTINE IN AN ANIMAL MODEL OF
PATERNAL NICOTINE EXPOSURE

A Dissertation Presented

By

MARKUS PARZIVAL VALLASTER

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 7th, 2017

INTERDISCIPLINARY GRADUATE PROGRAM

A Dissertation Presented By

MARKUS PARZIVAL VALLASTER

This work was undertaken in the Graduate School of Biomedical Sciences

(Interdisciplinary Graduate Program)

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This work is dedicated

To

Caroline and Josephine

With perpetual love and gratitude

Acknowledgements

I would like to express my gratitude to my mentor Oliver J. Rando, whose wealth of knowledge and rigorous scientific thinking have been a continuous stimulus to be curious, to strive for the most ambitious projects, design the best experiments, and subject the results to the most objective and stringent analyses. I hope this acquired trait has been passed on to my daughter.

I am grateful for all the discussion, scientific and non-scientific, that I had with Paul D. Gardner and Andrew R. Tapper. They introduced me to the magical field of neuroscience, which is astonishing to me every day anew. Paul has instilled in me an understanding of how great science can be, when it is taught right. I will always remember the laughs we had. Shackleton's map hangs right in front of me, while I am writing this.

Shweta, you made the experiments so much more fun, when you joined Ollie's lab in 2016. Your friendship means a lot to me.

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Abstract

Environmental conditions imposed onto organisms during certain phases of their life cycles such as embryogenesis or puberty can not only impact the organisms' own health, but also affect subsequent generations. The underlying mechanisms causing intergenerational phenotypes are not encoded in the genome, but the result of reversible epigenetic modifications. This work investigates in a mouse model the impact of paternal nicotine exposure on the next generation regarding addictive behavior modulation, metabolic changes, and molecular mechanisms. It provides evidence that male offspring from nicotine-exposed fathers (NIC offspring) are more resistant to lethal doses of nicotine. This phenotype is sex-specific and depends on short-term environmental challenges with low doses of nicotine prior to the LD₅₀ application. The observed survival phenotype is not restricted to nicotine as drug of abuse, but also presents itself, when NIC offspring are challenged with a cocaine LD₅₀ after acclimatization to low doses of either nicotine or cocaine. Functionally, NIC offspring metabolize nicotine faster than controls. Mechanistically, NIC offspring livers show global up-regulation of xenobiotic processing genes (XPG), an effect that is even more pronounced in primary hepatocyte cultures. Being known targets of Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR), these XPGs show higher baseline expression in naïve NIC offspring livers. Nicotine's action on the brain's reward circuitry does not appear to be of biological significance in our model system. Taken together, paternal nicotine exposure leads to a non-specific and conditional phenotype in male NIC offspring that may provide a general survival advantage against xenobiotic challenges.

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Figure 1.1. Action of nicotine on nAChRs within the reward circuitry of the brain

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RNA-Seq data are available at GEO, accession # GSE94059

ATAC-Seq data are available at GEO, accession # GSE92240.

File 2.1. RNA-Seq of TA and NIC hippocampus.

RNA-Seq data for hippocampus isolated from male TA or NIC offspring, with samples being collected from drug-naïve animals, from “chronic” animals subject to 6 days of nicotine administration prior to 24 hours of withdrawal, or subject to chronic nicotine exposure followed by a single sub-lethal injection of nicotine.

Link to dataset: <https://elifesciences.org/lookup/doi/10.7554/eLife.24771.019>

File 2.2. Hepatocyte RNA-Seq, naïve hepatocytes.

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CHAPTER I: INTRODUCTION

Smoking and Nicotine Addiction as Major Health Issues

Smoking has been a leading cause of cancer and ultimately death worldwide. Indeed, more than 480,000 people die in the United States each year as a result of smoking. It is estimated that more than 10% of these deaths are related to second-hand smoke exposure (CDC, 2014). Although the current opioid addiction crisis is much more present in the news and academic discourse, nicotine as the addictive component of tobacco poses a far greater risk to the general population for three major reasons:

1) Smoking causes or is associated with a variety of detrimental diseases such as cancers of the lung, larynx, esophagus, and even liver and colorectal cancer, or chronic diseases such as stroke, aortic aneurysm, coronary heart disease, Chronic Obstructive Pulmonary Disease (COPD), asthma, and male erectile dysfunction and infertility in both genders (CDC, 2014).

2) More people are addicted to smoking than to opioids and the age range is much wider than for other drugs of abuse (CDC, 2014). According to the American Society of Addiction Medicine (ASAM), the United States saw 52,404 lethal drug overdoses in 2015 with 20,101 of these deaths related to prescription pain medicine and 12,990 of these being heroin overdoses (CDC, 2015). This is an astonishing number and requires

the full attention of the medical community, but evanesces in the light of the death toll that is caused by smoking and nicotine addiction.

3) As many nicotine addicts display a relatively normal family life and enjoy greater social acceptance of their habits - to at least some extent - than opioid addicts, the risk of negatively influencing their social environment is much higher than with other drugs of abuse, which are generally seen as already dangerous and undesirable by society, especially when they are associated with intravenous application methods as in the case of heroin. The advent of electronic cigarettes has made this trend more pronounced in so far that inhaling nicotine vapor is less restricted and even more accepted than smoking cigarettes. The medical and public health community has yet to respond to this new nicotine application format.

It is, therefore, of pivotal importance to bring smoking, nicotine use, and nicotine addiction back into the focus of the public eye.

Mechanisms of Action of Nicotine

As stated above, tobacco use kills up to half of its users during their lifetime, resulting in a total worldwide death toll of nearly six million people per year (including ~600,000 non-smokers exposed to second-hand smoke) ((WHO), 2010; Cena et al., 2011; Changeux, 2010; Dome et al., 2010; Hurt et al., 2011; Prasad et al., 2009). If this negative trend continues, smoking will kill approximately 10 million people per year by 2020 (CDC, 2014). The addictive component of tobacco, nicotine, acts by stimulating nicotinic acetylcholine receptors (nAChRs), which are present not only in the CNS, but also in peripheral tissues such as muscle, lung, gastrointestinal tract, and testes (Dani and Bertrand, 2007; Gotti and Clementi, 2004; Grando et al., 2003; Kummer et al., 2006; Lips et al., 2007; Palmero et al., 1999; Schirmer et al., 2011; Wessler and Kirkpatrick, 2008). Nicotine binds these receptors as an agonist competing with endogenous acetylcholine, thus changing the protein and mRNA expression levels of nAChRs. In contrast to other competitive ligands, nicotine does not result in down-regulation of its receptor, but instead chronic nicotine exposure causes an up-regulation of nAChRs, which can be explained by desensitization of the receptor that is preceded by receptor activation (Fenster et al., 1999; Perry et al., 1999).

Nicotine causes the typical symptoms of reinforcement, tolerance, and dependence by acting within the mesostriatal and mesocorticolimbic dopamine (DA) pathways in the brain (D'Souza and Markou, 2011; Dani and Bertrand, 2007). Nicotine also activates

dopaminergic (DAergic) neurons in the ventral tegmental area (VTA), leading to DA release in the Nucleus Accumbens (NAc) and the prefrontal cortex (PFC). The mesocorticolimbic DA pathways, especially the electrochemical relays in the VTA and the NAc, contain a variety of nAChR subtypes that can be detected on a transcriptional level. These nAChR subunits are $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 2$, and $\beta 3$, all of which show high-affinity nicotine binding when incorporated into the cell membrane as a pentameric ion channel receptor (Azam et al., 2002). In addition, high density cell membrane levels of $\alpha 4$ and $\beta 2$ subunits have been shown on the protein level in DAergic neurons of the VTA, which play a particularly prominent role in the behavioral and rewarding effects of nicotine (Nashmi et al., 2003; Tapper et al., 2004). Nicotine's stimulation of the endogenous reward system through DA release causes positive feelings during smoking and reinforces addictive behavior. Conversely, nicotine withdrawal results in multiple negative symptoms consisting of both a somatic/physical component cued by the CNS and the peripheral nervous system (PNS), as well as an affective component, which might be due to the overstimulation of DAergic neurons in the reward circuitry during nicotine exposure and a corresponding desensitization of nAChRs that prevents physiologic DA release, when the exogenous stimulus has disappeared (Watkins et al., 2000). Additionally, the aversive effects of nicotine withdrawal are caused by Corticotropin-releasing factor (CRF) synthesis in DA-ergic neurons VTA that is released into the Interpeduncular Nucleus (IPN) (Grieder et al., 2014; Zhao-Shea et al., 2015).

Figure 1.1:

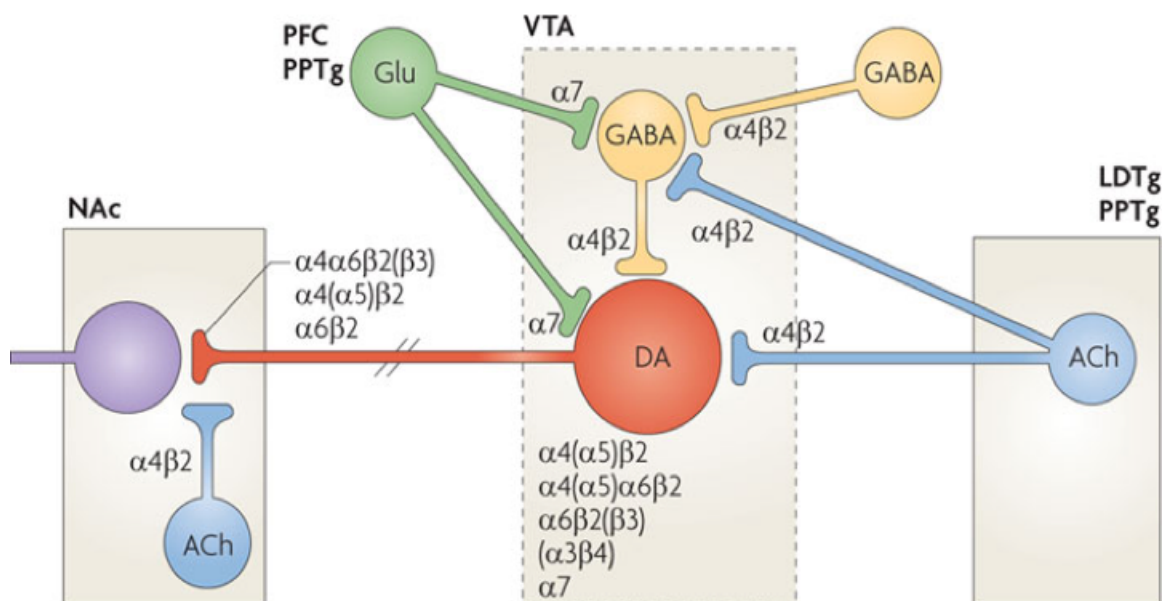


Figure 1.1. Action of nicotine on nAChRs within the reward circuitry of the brain.

Most neurons in the VTA are DA-ergic and receive excitatory stimuli from cholinergic neurons of the Laterodorsal Tegmental Nucleus (LDTg) and the Pedunculopontine Nucleus (PPTg). DA-ergic neurons of the VTA can also be stimulated by glutamatergic (Glu-ergic) neurons originating in different areas of the brain including the prefrontal cortex. Inhibitory signals are mainly mediated by γ -aminobutyric acid-ergic (GABA-ergic) neurons within the VTA itself. DA-ergic neurons of the VTA project to neurons within the NAc and receive regulatory stimuli from cholinergic neurons in the NAc. Nicotine can bind to various subtypes of pentameric nAChRs consisting of different α

and β subunits in these areas of the brain and stimulate dopamine release. Picture and legend adapted from (Changeux, 2010).

Addictive behavior, in general, is strongly correlated with social interactions, educational and economic status of the addict, and other socio-psychological trajectories. For example, the higher the educational status of an individual, the less likely is this individual to be a smoker (CDC, 2014). The transition from experimental smoker to regular smoker can be fluid and happens very quickly. The CDC states in its report that as few as 100 cigarettes can already push a person into the addictive realm. If a person starts smoking during the adolescence phase, this transition may happen faster (CDC, 2014). The adolescence phase is also a period, in which areas of higher cognitive function such as the prefrontal cortex, but also the amygdala and the limbic system, display increased developmental activity such as neuronal remodeling and synaptic pruning (Gogtay et al., 2004). This is true for the general acetylcholine system of these brain regions (Poorthuis et al., 2013), so the onset of addictive nicotine behavior during this developmentally vulnerable time in a person's life makes nicotine's interference all the more detrimental (Goriounova and Mansvelder, 2012). It is, however, also true that not everybody, who experiments with cigarettes during adolescence or any other time for that matter, becomes addicted to nicotine. Twin studies indicate a genetic component to nicotine-related behaviors such as smoking persistence, smoking quantity, and nicotine dependence (Carmelli et al., 1992; Heath and Martin, 1993; Li et al., 2003; True et al.,

1997). Furthermore, genetic variants influence the expression of nAChR subunits such as $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 3$ and $\beta 4$, which are associated with the amount of nicotine consumed in a given period of time, the intensity of a person's dependence on nicotine, as well as with diseases such as lung cancer, atherosclerosis and chronic obstructive pulmonary disease (COPD) (Berrettini et al., 2008; Bierut et al., 2007; Hurt et al., 2011; Saccone et al., 2009; Saccone et al., 2007; Thorgeirsson et al., 2008; Winterer et al., 2010). Other genetic contributors to nicotine biology include polymorphisms in genes involved in hepatic clearance of nicotine, such as the human cytochromes CYP P450 2A6 and 2B6 (Bloom et al., 2011; Johansson and Ingelman-Sundberg, 2011; Malaiyandi et al., 2005; Mwenifumbo et al., 2008; Nakajima et al., 2001; Sellers et al., 2003). As these subunits metabolize nicotine in humans, a slower or faster metabolic rate caused by polymorphisms can change the number of cigarettes smoked during a 24h period in order to keep nicotine levels sufficiently high in addicted individuals. However, all known genetic influences on nicotine-related behaviors explain only a small fraction of human heredity in nicotine usage (Johnson et al., 2010; Li et al., 2003; Maes et al., 2004). While cultural inheritance and rare SNPs could account for some of the missing heritability, an emerging hypothesis in complex diseases such as nicotine addiction is that inheritance of epigenetic information could also serve as a contributor.

Intergenerational Inheritance of Acquired Traits

The idea that environmental circumstances, in which an individual develops and grows up, can not only affect the health of this organism, but can also change the health prospects of future generations, a theory famously articulated by French biologist Jean-Baptiste Lamarck in his, at the time ridiculed, explanation as to why giraffes had evolved with such long necks, received renewed attention, when retrospective human cohort studies about the so called Dutch-Hunger-Winter were published in 2001 (Roseboom et al., 2001) and 2006 (Roseboom et al., 2006). During a certain, short period of time at the end of 1944 and beginning of 1945, the Netherlands suffered from severe food shortage and extreme famine with daily food portions dropping below 500 kilocalories per person at one point. Researchers looked at individuals, whose mothers were pregnant during this time of starvation, and found that those people had an increased risk of developing metabolic syndrome, glucose intolerance, and cardiovascular diseases 50 and 55 years after the occurrence of the event. The effects were more pronounced the earlier their mothers were in their pregnancies with the first trimester showing the strongest phenotype in the offspring. The scientist also discovered that these phenotypes correlated with hypo-methylation of the IGF-2 locus in these individuals (Roseboom et al., 2006; Roseboom et al., 2001). It is reasonable to imagine that individuals with IGF-2 hypo-methylation would utilize resources better in times of food shortage and famine, but have a disadvantage in terms of increased energy storage under normal living conditions,

which would in turn lead to all the metabolic disorders that were observed in the children of the Dutch Hunger Winter.

Another famous cohort study looked at harvest and food supply records of an isolated parish in northern Sweden, Överkalix, and correlated those with survival data and death records of the local church (Bygren et al., 2001; Kaati et al., 2002; Pembrey et al., 2006). An interesting finding here was that the time point of a certain exposure in one generation played a pivotal role in the transmission of epigenetic information to subsequent generations. For example, a male person's food supply before a crucial period of their adolescent development, the so called pre-pubertal slow growth period (SGP), usually between the ages eight and twelve, was inversely correlated with their grandson's longevity. In a similar manner, a grandmother's food supply in their early years from infancy to early puberty (zero to thirteen years of age), or rather dramatic changes in food supply during these years, affected their granddaughters' mortality and increased their risk to develop cardiovascular diseases. In all these analyses of correlations between grandparents and grandchildren, researchers observed a gender difference in the sense that grandmothers' exposure affected only granddaughters' phenotype and grandfathers' exposure affected only grandsons' phenotype. This gender difference, however, held also true when links between fathers and sons in terms of longevity and mortality were studied and data were normalized to a son's early life experiences as potential confounders such as whether his parents died, parental literacy status, family size, consanguinity on the grandfathers level, and what quantitative rank this son had amongst the total number of children, i.e., first born, second born, etc. (Kaati et al., 2007).

One of the major conclusions drawn from the Överkalix study was that the SGP is a particularly sensitive period during a human being's development that can not only affect themselves, but contribute to the health phenotype of future generations. To further test this hypothesis, the Avon Longitudinal Study of Parents and Children (ALSPAC) was designed (Pembrey et al., 2006). Researchers found, that a father's smoking habits of the past, even when adjusted for smoking at conception, are correlated with a son's, but not daughter's, body-mass-index (BMI) increase at the age of nine, again right during the critical SGP. The effects were greatest, when fathers started smoking very early, indeed, before the age of 11. The earlier the paternal onset of smoking, the greater the sons' BMI at age nine. At the age of 13 and up, sons from fathers, who started smoking before the age of eleven, had an average of five to ten kilogram more fat mass (Northstone et al., 2014). For our experimental design of paternal nicotine exposure in a mouse model and the concomitant hypothesis behind it, it is worth noting, that in the ALSPAC study a very specific exposure or stimulus in the paternal generation was associated with a change in a very non-specific or general metabolism rate in the F1 generation.

Since then, a plethora of studies on epigenetic inheritance have been published, so that we can now define epigenetic inheritance as the inter- or transgenerational transmission of information beyond the DNA sequence, which includes DNA methylation, histone modification, small RNA populations, and other entities that can be preserved during the fertilization process and have the ability to influence its outcome. The majority of examples of intergenerational (two generations involved) and trans-generational (more than two generations involved) information transfer in mammals concern maternal

influences on offspring (Harris and Seckl, 2011), including in-utero passage of photoperiod information in various rodents (Horton, 2005; Varcoe et al., 2011), cultural inheritance of stress reactivity, and maternal grooming behavior in rats (Champagne and Meaney, 2001; Champagne et al., 2004; Champagne et al., 2003; Fish et al., 2004; Meaney et al., 2007; Weaver et al., 2004), as well as metabolic and psychiatric sequelae of fetal malnutrition in humans and rodents (Bush and Leathwood, 1975; Hales and Barker, 2001; Maekawa et al., 2011; Schulz, 2010; Stein et al., 2004), multigenerational effects of treating pregnant females with endocrine disruptors (Alonso-Magdalena et al., 2011; Gaspari et al., 2011; Guerrero-Bosagna et al., 2010; Guerrero-Bosagna and Skinner, 2009; Kang et al., 2011; Masuo and Ishido, 2011; Skinner et al., 2011), and altered vitamin intake (Burdge et al., 2009; Smedts et al., 2009; Smedts et al., 2008). Since the *in-utero* environment itself could have effects on the offspring without any necessity for transfer of transgenerational information via the germline, multigenerational observations have to be performed in order to exclude direct effects of the environment on the fetus. For example, if a pregnant woman smokes or drinks alcohol during her pregnancy, harmful toxins can directly reach the fetus via the placenta as, for example, in the so called fetal alcohol syndrome. Other more indirect effects that still have an immediate impact on the developing fetus are changes in liver metabolism, increased blood pressure of the mother, or stimulation of the intrinsic inflammatory system by, once again, alcohol and cigarette smoke (Bakhru and Erlinger, 2005). In the study about inflammatory response and smoking, researchers found that it took participants in the study 5 years after they had stopped smoking to see their inflammatory biomarkers (C-

reactive protein or CRP) within normal range again, which is approximately the same time period that is required to reduce a smoker's cardiovascular risk (Dobson et al., 1991). This fact is of particular interest, since chronic inflammation responses in themselves may exert transgenerational transmission potential that could confound findings in intergenerational smoking studies even in cases, in which test subjects had stopped smoking before conception, depending on the length of time that has passed between those two events.

It is important to note that fathers often provide little more than sperm to their offspring, making paternal effects of the environment an ideal approach to studying intergenerational inheritance. It has recently been shown that paternal low protein diet indeed affects hepatic expression of cholesterol-related genes in offspring (Carone et al., 2010). These findings were correlated with hypo-methylation of an enhancer sequence upstream of Peroxisome Proliferator-Activated Receptor Alpha (PPAR α), a Nuclear Hormone Receptor (NHR), which is involved in the regulation of lipid metabolism. This is consistent with the epidemiological findings of the Överkalix and ALSPAC studies as described above. As the paternal generation of mice was exposed to low protein diet, it is not too surprising that the offspring phenotype displayed changes in lipid metabolism. Both the type of paternal exposure, as well as the response in offspring can be described as non-specific interference with general nutrient metabolic pathways in an organism. Therefore, we designed a follow-up study of paternal nicotine exposure using a well known receptor-ligand interaction to investigate, how specific an intergenerational

phenotype could be that responds to an environmental challenge in the paternal generation.

Specific v. Non-specific Intergenerational Inheritance

There are, generally speaking, two schools of thought on what influence the exposure to certain environmental conditions or the change of the same may have on offspring phenotype. The vast majority of inter- or transgenerational inheritance studies fall in either one of these categories.

Firstly, the offspring response could be non-specific in nature to the parental stimulus. In this scenario, subsequent generations would react to a “quality-of-life” event that the parental generation experienced in a way that either benefitted the parents or adversely affected their overall living conditions. We would, therefore, expect to see a change in phenotypes that would make the offspring abler to adapt and adjust to the potential recurrence of these parental life events. These phenotypes could include alterations in metabolic pathways such as lipid storage, glycolysis, gluconeogenesis, etc. Indeed, most inter- and transgenerational studies see changes such as these in offspring. The Dutch Hunger Winter analysis described above is a perfect example. The phenotypic outcome in first generation children from mothers, who had been exposed to severe famine during pregnancy was altered glucose metabolism, metabolic syndrome, diabetes, and cardiovascular diseases associated with an up-regulation of IGF-2. An improved utilization of nutrients in terms of up-regulation of metabolic pathways would certainly

come in handy, when there is shortage of food, but can be detrimental in circumstances, when there is plenty of food around.

On the maternal side of inter- and transgenerational inheritance, this phenomenon, that the intrauterine environment plays a crucial role in the development of the fetus and shortages in this environment can prepare the offspring for poor living conditions later in life by priming metabolic pathways in a more efficient way, was described by David J. P. Barker in 1992 and has since become known as the Barker Hypothesis or Thrifty Phenotype Hypothesis (Hales and Barker, 1992, 2001). The medical community knows well that babies, who are too small for their gestational age (SGA), i.e. below the 10th percentile at birth, due to intrauterine growth restrictions (IUGR) can develop metabolic syndrome later in life. This phenotype is associated with alterations of growth and proliferation pathways of IGF-1 (Wallborn et al., 2010). These findings of altered metabolic pathways in offspring as a response to adverse *in-utero* conditions have been replicated multiple times in animal models of protein restriction (20% v 8%) (Fernandez-Twinn et al., 2005; Ozanne et al., 2003), caloric restriction (Jimenez-Chillaron et al., 2009), or placental insufficiency (Simmons et al., 2005; Simmons et al., 2001).

On the father's side, our very own lab has shown that paternal exposure to low-protein diet in a mouse model affects cholesterol and lipid metabolism in F1 offspring (Carone et al., 2010). Another rather remarkable study that shall be mentioned here looked at a more specific exposure paradigm in fathers by using carbon tetrachloride to induce liver damage in male rats rather than a diet-based approach (Zeybel et al., 2012). When the offspring was exposed to the same chemical compound again, the researchers noticed that

these animals displayed improved hepatic healing in a progressive manner over generations to the extent that carbon tetrachloride exposure to the F3 generation did not result in any liver cirrhosis at all. These findings were restricted to male offspring only. The wound healing phenotype correlated with an up-regulation of PPAR γ and down-regulation of Transforming Growth Factor β 1 (TGF- β 1), which is known to be pro-fibrogenic.

Taken together, all these examples of inter- and transgenerational inheritance in human and animal studies point toward a more generalized, less specific offspring response to parental exposure paradigms. It shall, however, be noted that these studies only look at metabolic pathways. We cannot decide whether this is, because the researchers did indeed look at other phenotypes, as well, but did not report on them, as there was no difference between groups, or because other phenotypic read-outs such as behavior or specific compound responses were not considered in the first place, even in the case of the wound-healing study, which again reported a rather non-specific offspring response.

There are a few exceptions to this, one of them being a study that came out of the Mansuy laboratory in Switzerland (Gapp et al., 2014). Here, researchers subjected male mice to a daily regimen of maternal separation combined with unpredictable maternal stress (MSUS) from postnatal day 1 to postnatal day 14. Offspring were obtained by mating either MSUS males or control males with control females. The peculiarity of this paradigm is that both metabolic and behavioral responses were tested in MSUS and control offspring. MSUS F1 and, remarkably, also F2 male offspring displayed reduced

fear and avoidance in the elevated-plus maze and light-dark box experiments. These results were combined with an analysis of F1 and F2 blood insulin and glucose levels at baseline and post corticosteroid injection to mimic a stress response. MSUS offspring displayed insulin hyper-sensitivity and hyper-metabolism, with the latter being present only in F2 males. Separation stress and anxiety, on the other hand, are again very non-specific stimuli that could act on many different receptors in many different organs and tissues in the body from liver to brain and anything in between. In human studies, a large Japanese human cohort study found that children with SGA displayed behavioral abnormalities at the age of eight such as attention problems and aggressive behavior towards others (Takeuchi et al., 2017), as well as difficulties in performing independent tasks such as walking or using a spoon to eat at the age of two and a half years (Takeuchi et al., 2016).

Thus, although the Mansuy and Japanese cohort studies are rather unique in their read-out by using behavioral tests and metabolic analyses, the stimuli applied to the parental generation are still general. Therefore, it cannot be concluded from any of these studies that epigenetic inheritance of acquired traits follows either a “quality-of-life” paradigm beyond what was tested here or could also be more specific depending on the stimulus.

Secondly, the offspring response could be specific in nature to the parental stimulus. The number of studies that try to expose parents to a more specific stimulus, i.e., choosing a compound that elicits, for example, a specific receptor-ligand response, is astonishingly

small. There is, however, one study that stands out (Dias and Ressler, 2014). Here, researchers used an adverse odor, acetophenone, to stimulate a specific olfactory receptor, *Olf151*. The experimental design and flaws of this study will be addressed in the discussion section of this work. For now, it suffices to say that the scientists found that subsequent mouse generations reacted more sensitively to acetophenone, when their fathers had been exposed to this odor as well. This behavioral finding was associated with hypo-methylation of *Olf151* in sperm from the paternal and naïve male F1 generations.

Besides this particular study, there is not much else known about specific inter- or transgenerational interaction studies. In other experiments, researchers exposed the parental generation to endocrine disruptors such as Methoxychlor (Muroso and Derk, 2005), the fungicide Vinclozolin (Uzumcu et al., 2004), and Bisphenol-A (Maffini et al., 2006). These endocrine disruptors are of particular interest, as they have been shown to cause sub-fertility in men (Den Hond et al., 2015). Although all these chemical compounds can bind to receptors in the body, they can also be non-specific in their interactions with metabolizing enzymes such as cytochrome P450. For example, Bisphenol-A interferes with estrogen signaling by binding to ERR- γ in a variety of tissues, amongst them the placenta with a high level of ERR- γ expression (Takeda et al., 2009; Tohme et al., 2014), thus affecting sexual differentiation, behavior, and gene expression (Ilagan et al., 2017; Kundakovic et al., 2013). In the case of Bisphenol-A, the binding partner is clear. In other cases such as Methoxychlor and Vinclozolin, the entities involved in binding are less defined or less determined. Glucocorticoid receptors have

been implicated in these interactions (Zhang et al., 2016), as have gap junction proteins such as connexin-43 and the activation of MAP kinase pathways (Babica et al., 2016). The read-out in all these studies is rather metabolism-centric, and does not involve any behavioral experiments.

In summary, without taking the Olfr151 exception into account, even experimental paradigms that expose the parental generation to a more specific stimulus cover a wide range of receptors and pathways and cannot serve as specific receptor-ligand interaction studies.

We, therefore, sought to embark on the adventure to investigate a receptor-ligand interaction (nicotine – nAChRs), which is truly specific indeed, and perform extensive phenotype characterization of the F1 offspring on both behavioral and metabolic levels.

Study Goal and Hypothesis

The goal of this study is to determine whether paternal exposure to a specific environmental compound leads to a specific phenotype response in offspring or a more generalized change in offspring metabolism.

We hypothesize that paternal nicotine exposure will lead to a phenotype in offspring from nicotine-exposed fathers that is

- 1) distinctly different from what is seen in control offspring and
- 2) nicotine-specific either in terms of tissue or organ preference or in terms of functional outcome such as altered behavior.

By using nicotine, we are able to investigate a specific receptor (nAChR) ligand (nicotine) interaction that has the potential to elicit an F1 phenotype that is specific to nicotine, possibly mediated through the very same receptors. We use a mouse model of paternal nicotine exposure, as it allows us to control for confounders such as social and environmental interactions, food intake, or sleep-wake cycle more rigorously. The scope of this investigation is an extensive analysis of the F1 generation on a phenotypic and, as far as possible, on a mechanistic level, not how this information about nicotine exposure could potentially be transferred from fathers to offspring. We will describe a variety of

behavioral and phenotypic tests that enable us to systematically characterize the phenotypic observations that we make in F1 offspring from nicotine-exposed and control fathers. We will also propose a potential mechanism that could be responsible for the observed phenotype within the F1 generation. We will not investigate nor speculate on potential mechanisms that could be responsible for the preservation of this information through the paternal germline over generations. We hope that we can, with this study, contribute to the understanding of phenotypic responses in offspring as a function of intergenerational inheritance.

CHAPTER II: STUDY REPORT

Preface

The body of this work is reprinted from the following work:

Paternal nicotine exposure alters hepatic xenobiotic metabolism in offspring

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Contributions:

Markus P. Vallaster (MPV) performed behavior experiments and phenotypic profiling in mice (Fig. 2.1, 2.3, 2.4, 2.5, 2.6), toxicity study (Fig. 2.7, 2.12, 2.13). Rubing Zhao-Shea (RZS) was responsible for the elevated-plus maze study (Fig. 2.2). Cotinine levels post nicotine injection were collected, measured, and analyzed by MPV (Fig. 2.9A). Shweta Kukreja (SK) executed glucose and insulin tolerance tests, pRT-PCR validation of *Cyp2a5*, *Scdf1*, and *Apoa2*, and analyzed the data (Fig. 2.10). Hepatocyte isolation and culture were performed by MPV (Fig. 2.9B). MPV and SK collected and processed *in-vivo* and *in-vitro* liver samples for RNAseq (Fig. 2.9C-E). MPV dissected tissue, made RNAseq libraries, and analyzed RNAseq data from brain regions (Fig. 2.8A-B). Samples for ATACseq (Fig. 2.9F, Fig. 2.11, were collected by MPV and SK, processed by SK, and analyzed by Xin Y. Bing (XYB). Tissue dissection of the hippocampus region was performed by MPV (Fig. 2.8C-E). Hippocampus was processed for immune-histological staining and resulting data were analyzed (Fig. 2.8C-E) by Jennifer Ngolab (JN). Livers for immune-histological assessment of apoptosis (Fig. 2.14) were dissected by MPV, stained by the Pathology Core Facility of University of Massachusetts Medical School, and analyzed by MPV. Paul D. Gardner, Andrew R. Tapper, and Oliver J. Rando supervised the project, provided conceptual input, and analyzed the data. The manuscript was originally written by MPV and reviewed and edited by ART and OJR.

Abstract

Paternal environmental conditions can influence phenotypes in future generations, but it is unclear whether offspring phenotypes represent specific responses to particular aspects of the paternal exposure history, or a generic response to paternal “quality of life”. Here, we establish a paternal effect model based on nicotine exposure in mice, enabling pharmacological interrogation of the specificity of the offspring response. Paternal exposure to nicotine prior to reproduction induced a broad protective response to multiple xenobiotics in male offspring. This effect manifested as increased survival following injection of toxic levels of either nicotine or cocaine, accompanied by hepatic up-regulation of xenobiotic processing genes, and enhanced drug clearance. Surprisingly, this protective effect could also be induced by a nicotinic receptor antagonist, suggesting that xenobiotic exposure, rather than nicotinic receptor signaling, is responsible for programming offspring drug resistance. Thus, paternal drug exposure induces a protective phenotype in offspring by enhancing metabolic tolerance to xenobiotics.

Introduction

Environmental conditions experienced in one generation can affect phenotypes that manifest in future generations, a phenomenon sometimes referred to as the “inheritance of acquired characters.” In mammals, a substantial body of literature links various maternal exposures to offspring phenotypes (Harris and Seckl, 2010; Rando and Simmons, 2015; Simmons, 2011), and an increasing number of studies have shown that *paternal* environment can also alter offspring phenotype (Rando, 2012). Paternal effect paradigms are of particular mechanistic interest in mammals, given that it is challenging to disentangle maternal environment effects on the oocyte epigenome from effects on uterine provisioning during offspring development. In contrast, in many paternal effect paradigms, males contribute little more than sperm to the offspring, simplifying the search for the mechanistic underpinnings of paternal effects on children. A large number of paternal exposure paradigms have been used to show that a father’s diet can affect metabolic phenotypes in the next generation (McPherson et al., 2014; Rando, 2012), while another large group of studies link paternal stress (using paradigms such as social defeat stress, or early maternal separation) to anxiety-related behaviors and cortisol release in offspring (Bale, 2015). Finally, a growing number of toxins and drugs have been shown to induce effects on various offspring phenotypes (Skinner et al., 2011; Vassoler et al., 2013; Yohn et al., 2015; Zeybel et al., 2012).

A key challenge of such studies at present is to understand how the offspring phenotype is related to the stimulus presented in the paternal generation – in other words, how specific is the offspring response? This challenge is compounded by the fact that many of the stimuli used for paternal effect paradigms – low protein and high fat diets, social stressors, and endocrine disruptors – have pleiotropic effects on organismal physiology. We therefore sought to develop a paternal effect paradigm based on a defined ligand-receptor interaction, to enable pharmacological interrogation of the specificity of the offspring phenotype. Nicotine is a commonly-used drug in humans, and acts by binding to and activating nicotinic acetylcholine receptors (nAChRs), ligand-gated cation channels normally activated by the endogenous neurotransmitter acetylcholine. Maternal use of nicotine has been linked to multiple phenotypes in offspring (Yohn et al., 2015; Zhu et al., 2014), and although effects of paternal nicotine exposure have been less-studied, paternal smoking in humans has been suggested to affect metabolic phenotypes in children (Pembrey et al., 2006).

Here, we develop a rodent model for paternal nicotine effects, asking 1) whether exposure of male mice to nicotine could impact phenotypes in offspring, and 2) whether any affected phenotype would be specific for nicotine. We found that paternal exposure to nicotine induced a protective response in the next generation, as male offspring of nicotine-exposed fathers exhibited significant protection from nicotine toxicity. Importantly, this toxin resistance was not specific to nicotine, instead reflecting a more general xenobiotic response – offspring of nicotine-exposed fathers exhibited increased hepatic expression of a variety of genes involved in clearance of xenobiotics, and these

animals were resistant to cocaine as well as to nicotine toxicity. Finally, we found that enhanced resistance to nicotine toxicity was also observed in offspring of males treated with the nicotine antagonist mecamylamine, strongly suggesting that drug resistance in offspring is a common outcome of paternal exposure to multiple xenobiotics rather than a specific response arising from nicotine signaling. Taken together, our results describe a novel paternal effect paradigm, and demonstrate that in the case of paternal nicotine exposure, the phenotype observed in offspring is a relatively generic response – enhanced xenobiotic resistance – rather than a selective downregulation of the specific molecular pathway subject to paternal perturbation.

Results

Effects of paternal exposure history on offspring nicotine sensitivity

We established a paternal exposure paradigm in which male mice were either provided with nicotine hydrogen tartrate (nicotine 200 µg/ml free base, sweetened with saccharine) in their drinking water, or a control solution of tartaric acid and saccharine. Mice consumed nicotine or control solutions (NIC or TA, respectively) from 3 weeks of age until 8 weeks of age. As previously described (Zhao-Shea et al., 2015), this administration regimen maintains a high level of nicotine in the bloodstream (**Figure 2.1, panel A-B**), and results in nicotine dependence in exposed animals (Zhao-Shea et al., 2013). Males were then withdrawn from nicotine for one week prior to mating in order to prevent any potential for seminal fluid transmission of nicotine (the half-life of nicotine in mice is ~10 minutes, the half-life of its “long-lived” metabolite cotinine is ~40 minutes (Siu and Tyndale, 2007)). Nicotine and control males were then mated with control females. Overall, we observed no difference in average size or sex ratio of litters arising from control or nicotine matings, or in offspring body weights (**Figure 2.1, panel C-F**).

Figure 2.1:

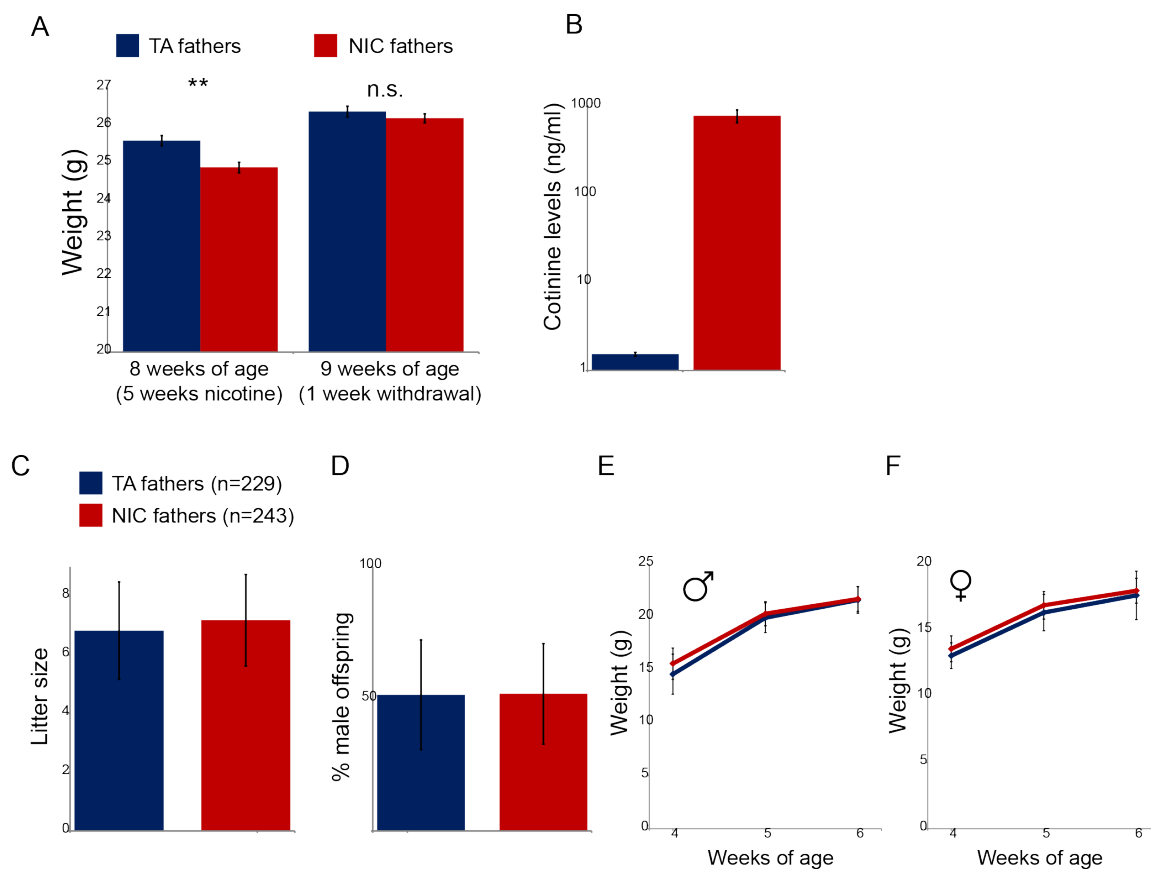


Figure 2.1. Physiological effects of nicotine exposure on treated males.

(A) Weight of males subject to 5 weeks of exposure to nicotine (NIC) or control (TA) solution. Data are shown for animals at the end of 5 weeks of nicotine exposure (** indicates $p < 0.01$), and following a week of withdrawal (n.s.: not significant), as indicated.

(B) Blood levels of cotinine, a relatively long-lived metabolite of nicotine, in males (at 8 weeks of age, following 5 weeks of nicotine/control treatment) consuming control or nicotine solutions.

(C) Average litter size for offspring of control and nicotine-treated males. Sample size reported as number of litters. Data show average plus/minus S.E.M.

(D) Average gender ratio for offspring of control and nicotine-treated males. Sample size reported as number of litters. Data show average plus/minus S.E.M.

(E-F) Average weights for male (E) and female (F) TA and NIC offspring at 4, 5, and 6 weeks of age. Data are shown as average plus/minus S.D.

We first sought to determine whether the enforced nicotine withdrawal in our exposure paradigm might result in a paternal stress response that could affect the phenotype of progeny. As anxiety-related behaviors have been reported in offspring of males subject to several distinct stress paradigms (Dietz et al., 2011; Gapp et al., 2014; Short et al., 2016) (albeit not all such paradigms – (Rodgers et al., 2013)), we therefore assessed anxiety behaviors in TA and NIC offspring. Importantly, we observed no differences between TA and NIC offspring in time spent in the center during an open field anxiety test, or in time spent or number of entries into the open arms of an elevated plus maze (**Figure 2.2**).

These results and results discussed below (see **Figure 2.13**) indicate that our nicotine administration paradigm does not induce a stress response robustly enough, or for long enough prior to mating, to affect offspring phenotype.

Figure 2.2:

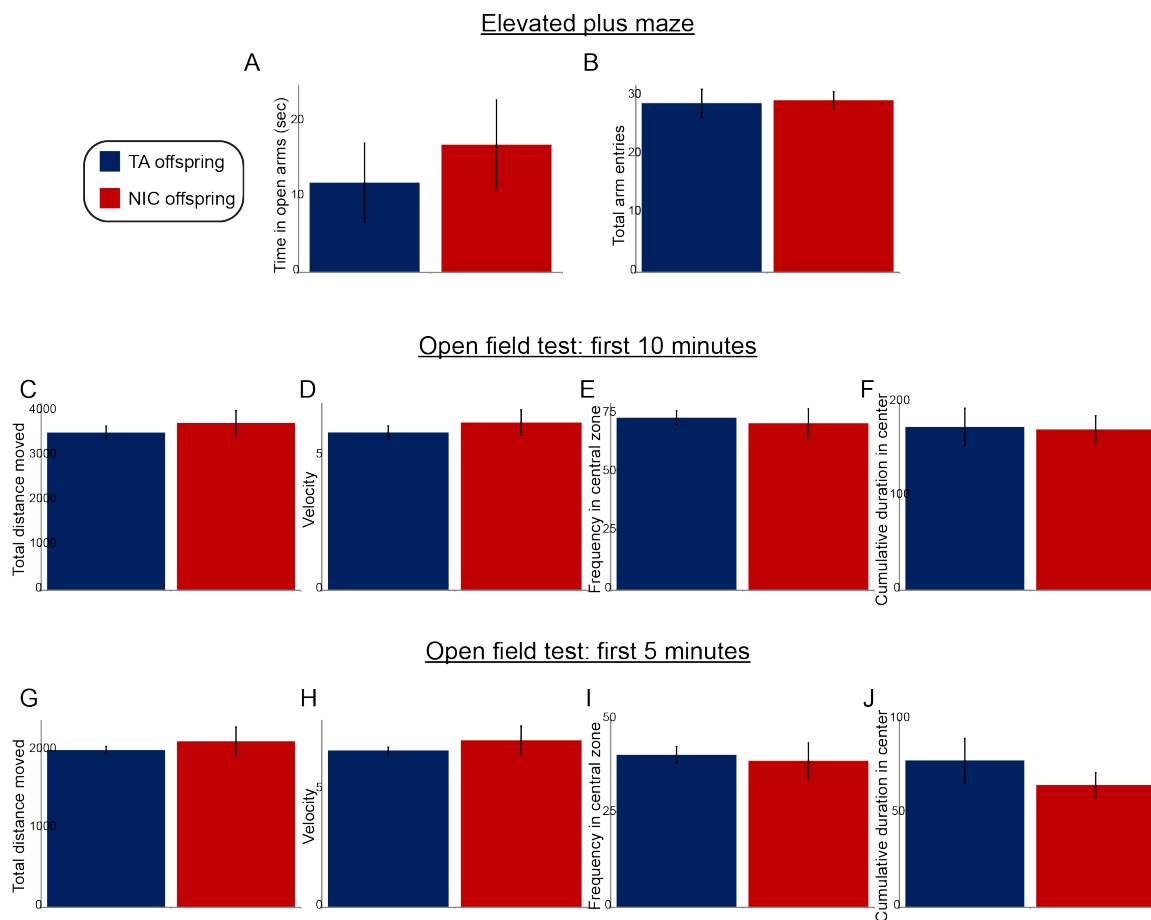


Figure 2.2. Paternal nicotine exposure does not affect offspring anxiety-related behaviors.

(A-B) Data are shown for elevated plus maze performance – time spent in open arms (A), or total entries into the open arms (B) – for TA offspring (n=7) and NIC offspring (n=11). (C-J) Open field test performance, shown for the first 10 minutes (C-F) or first 5 minutes (G-J) following introduction of the animal into the enclosure. Panels show total distance

moved (C,G), velocity (D,H), fraction of time spent in the center of the open field enclosure (E,I), and cumulative time spent in the central zone (F,J).

All data show average \pm S.E.M. None of the differences between TA and NIC offspring are statistically significant.

We next asked whether paternal nicotine administration could more specifically affect nicotine-related phenotypes in the next generation. We first focused on a physiological readout of offspring sensitivity to nicotine, using a well-established assay for suppression of locomotor activity by acute nicotine administration (Tapper et al., 2004). Briefly, after acclimating animals to a saline injection protocol for three days, animals are injected with either nicotine (1.5 mg/kg) or saline, and immediately introduced to a novel environment. Saline-injected animals actively explore the novel environment, and locomotor activity is quantified over a 40-minute time course (**Figure 2.3** – Baseline). In this paradigm, injection of nicotine results in rapid suppression of locomotor activity, followed by a gradual recovery of exploratory behavior over the time course of the assay. Using this assay, we observed no significant difference in nicotine sensitivity between TA and NIC offspring, either for male or female offspring (**Figure 2.3**, **Figure 2.4**). We therefore conclude that the acute locomotor suppression response to nicotine is not altered by our paternal nicotine exposure paradigm.

Figure 2.3:

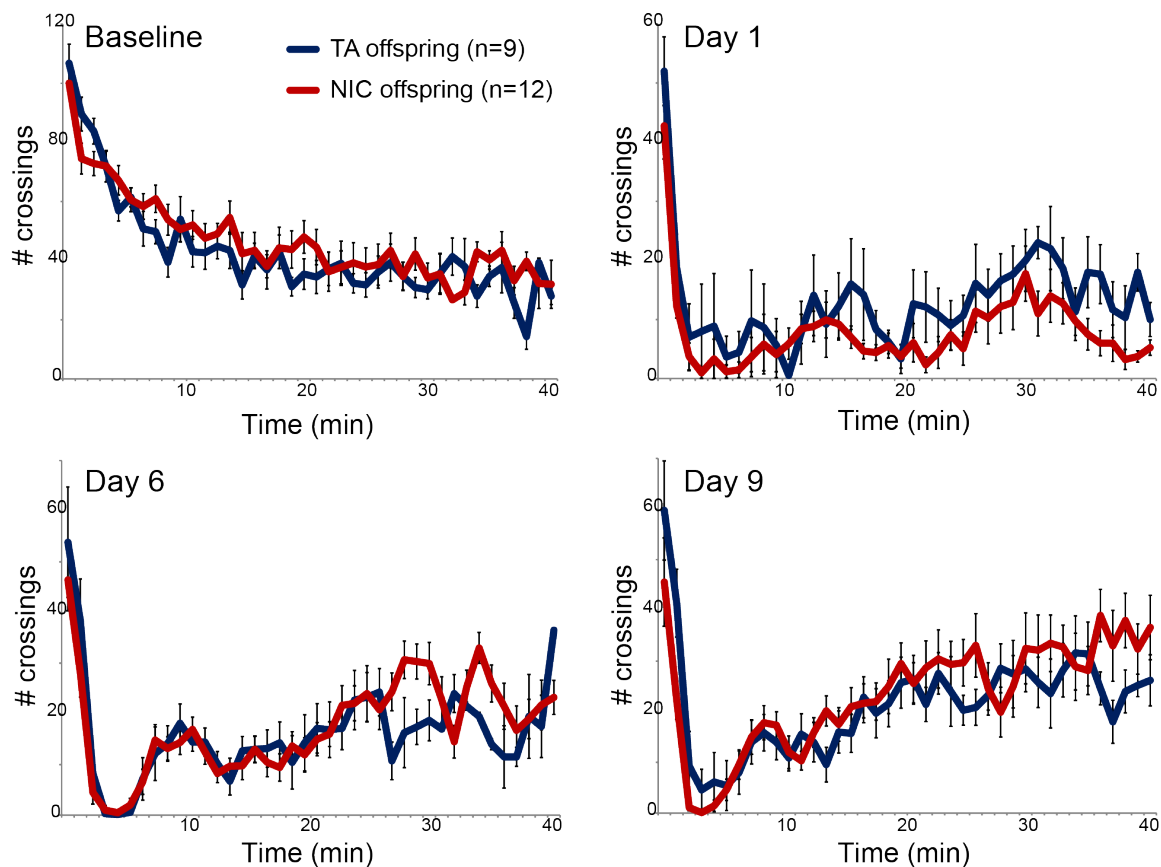


Figure 2.3. Nicotine suppression of locomotor activity is unaffected by paternal nicotine history.

Nicotine effects on locomotor activity were assayed in male offspring of control (TA) or nicotine-exposed (NIC) fathers. Data for females and alternative administration regimens are shown in **Figure 2.4**. For each plot, males were injected with either saline or nicotine immediately prior to being placed in a novel environment for 40 minutes, during which locomotor activity was assessed by the number of times the animal interrupted a light beam during each minute. Each time point shows the number of beam crossings in that

minute, shown as average plus/minus S.E.M. for all animals tested. Importantly, here and throughout the manuscript, the listed number of animals represent the number of litters analyzed, as we only assess one animal per litter in a given assay. Data are shown for saline injection (“Baseline”) – exploratory behavior decreases over time in saline-injected animals as they habituate to the locomotor cage – and for 1.5 mg/kg nicotine injection in animals naïve to nicotine (Day 1) or following five or eight prior days of the same nicotine injection and locomotor assessment protocol.

Figure 2.4:

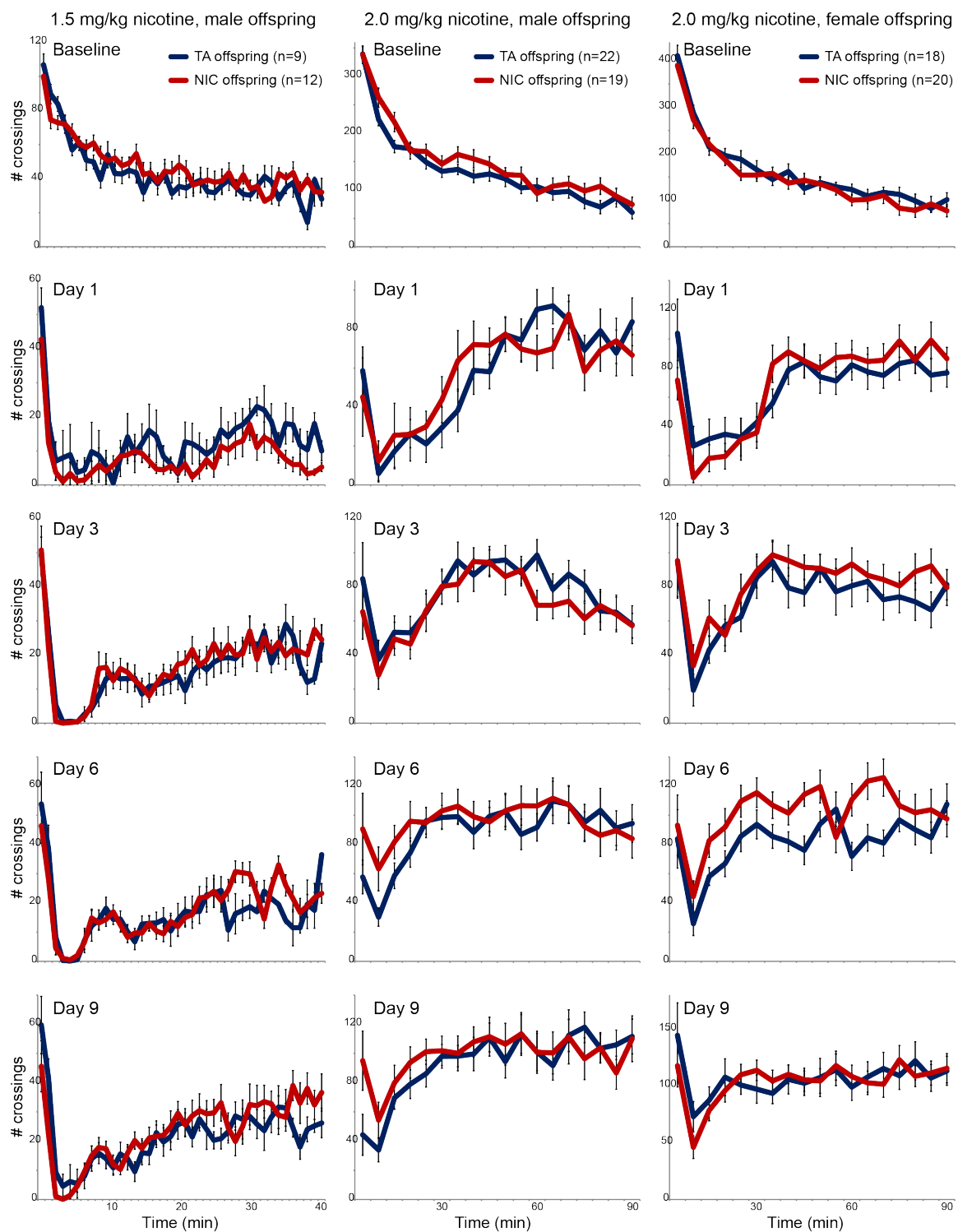


Figure 2.4. No significant effects of paternal nicotine exposure on offspring locomotor response to nicotine.

For each column, offspring of control and nicotine-treated males (TA and NIC, respectively) were subject to a locomotor activity assay as follows. Animals were first acclimated to intraperitoneal saline injections once a day for three days. On day three (Baseline), offspring were injected with saline, then placed in a novel environment – a box equipped with infrared photodiodes to enable detection of locomotor activity. Saline-injected animals actively explore the novel environment, and locomotor activity is quantitated over a 40- or 90-minute time course by the number of times the animal interrupts the light beam. Exploratory behavior decreases over time in saline-injected animals as they fully explore the enclosure. On nine subsequent days (data for four representative days are shown in each column), animals are injected with nicotine (1.5 or 2.0 mg/kg, as indicated) and immediately introduced to the measurement box. In this paradigm, injection of nicotine results in rapid suppression of locomotor activity, followed by a gradual recovery of exploratory behavior over the time course of the assay. Data here are shown as mean plus/minus S.E.M.

We next sought to identify any effects of paternal nicotine exposure on nicotine reinforcement in offspring using an operant self-administration assay (Fowler et al., 2011). Here, after surgical implantation of a catheter into the superior vena cava, animals are subject to caloric restriction and trained to nose-poke an active portal to self-

administer (SA) sucrose. TA and NIC offspring exhibited similar behavior during the training period, with the exception of a modest albeit significant difference in sucrose SA on the final day of dietary training (**Figure 2.5**).

Figure 2.5:

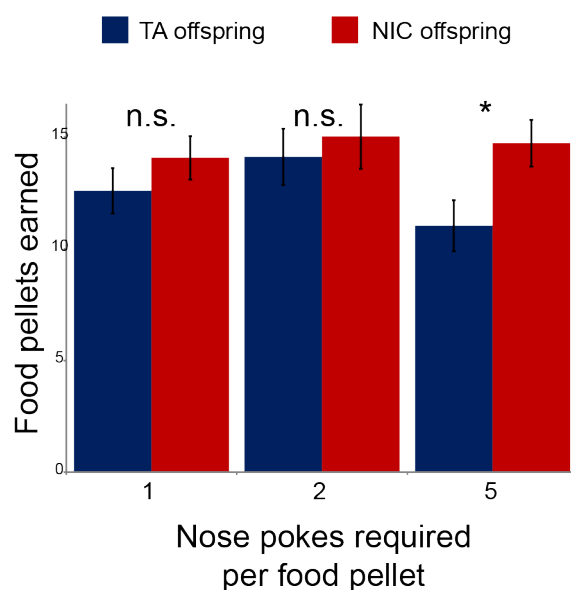


Figure 2.5. Modest effect of paternal nicotine exposure on dietary training.

Following surgical implantation of a central line, TA and NIC offspring were allowed to recover for 3 days. Animals were then subject to caloric restriction (80% of daily diet w/w compared to animals feeding *ad libitum*), placed in a self-administration box with two buttons, one of which was marked with a small light. Animals were then provided with sucrose pellets in response to a nose poke on the lit button – for 3 days a pellet was provided following each correct nose poke, then for one more day two nose pokes were required for a pellet, and finally five nose pokes were required for a food pellet for 3 days. Bars here show the number of food pellets earned in one hour for TA and NIC

offspring – NIC offspring earned moderately more sucrose pellets in the final reward regime than TA offspring ($p=0.03$). This enhanced food training carried over to the first day of nicotine self-administration (**Figure 2.6A**), when NIC animals self-administered slightly more nicotine than TA animals, but this difference only persisted for the first day.

After seven days of food shaping, animals were placed in the operant chamber, a nicotine infusion pump was connected to the central catheter, and the dietary reward for nose-poking the active portal was replaced with a nicotine infusion. The amount of nicotine self-administered every day was then measured per session over the course of 35 days, with the nicotine infusion dose increasing every 4-8 days (**Methods**). Overall, there was no difference in daily nicotine SA between offspring of control males and offspring of nicotine-exposed males (**Figure 2.6A**), indicating that nicotine reward behavior is not significantly reprogrammed by our paternal exposure paradigm.

Offspring of nicotine-treated males exhibit enhanced resistance to nicotine toxicity

Nonetheless, a clear phenotype emerged serendipitously from the SA paradigm. We found that in our strain background, the escalating nicotine dosing schedule of SA resulted in death of nearly all animals tested at the highest doses used. Surprisingly, NIC offspring survived for many more days, on average, than TA offspring (**Figure 2.6B**). This difference in survival was highly significant (Gehan-Breslow-Wilcoxon $p < 0.0001$). As there was no difference in the daily levels of nicotine administered by either group

(**Figure 2.6A**), this result suggests that paternal nicotine exposure can protect offspring from nicotine toxicity.

Figure 2.6:

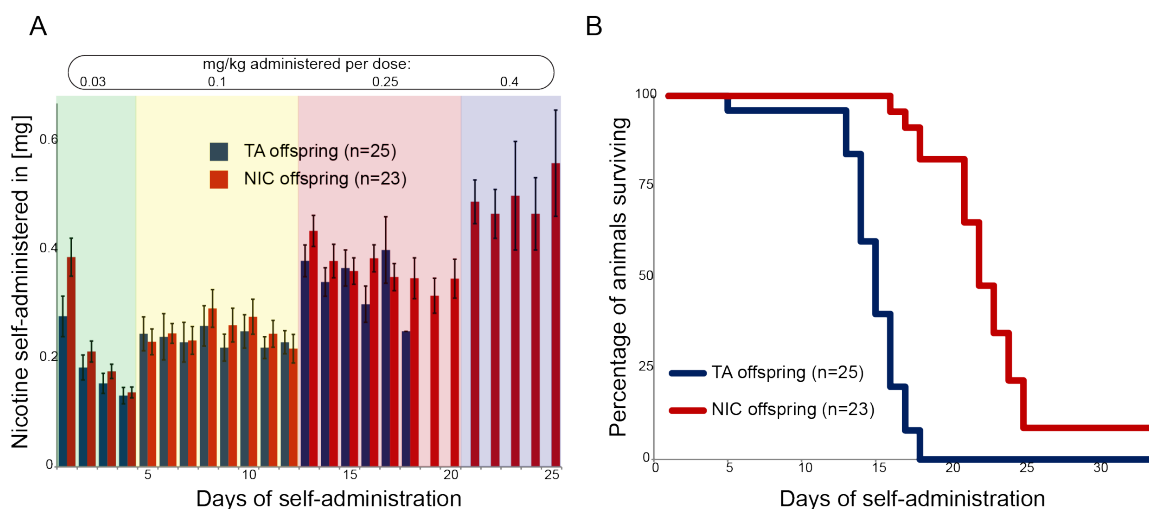


Figure 2.6. Paternal experience affects nicotine toxicity, but not self-administration, in offspring.

(A) Paternal nicotine exposure does not affect nicotine self-administration in offspring. Each day, a mouse trained to self-administer nicotine (**Methods**) was connected to the self-administration apparatus for one hour, with the dose of nicotine administered via cannula for every correct nose poke ramping up every 4-8 days, as indicated. Total nicotine self-administered is shown for each day of the protocol as average and S.E.M. Note that the numbers of animals participating in the trial decreased over time due to removal from the protocol (clogged catheter) or death – the listed n represents all animals that remained on the protocol until death.

(B) Offspring of nicotine-exposed fathers exhibit significant protection from nicotine toxicity. Survival curve is shown for all animals on the self-administration protocol (underlying data are provided in accompanying Source Data file). Nicotine offspring exhibited significantly increased survival during the time course of the assay relative to control offspring (Kaplan-Meyer survival curve, $P < 0.0001$ for both Log-rank test and Gehan-Breslow-Wilcoxon test).

As TA and NIC offspring exhibit differences in their resistance to lethal doses of nicotine despite no difference in the daily level of nicotine consumed, we asked whether the effect of paternal nicotine exposure on offspring survival could be recapitulated using a single dose nicotine challenge, rather than the laborious self-administration protocol described above. This nicotine challenge was performed using two distinct paradigms. First, we simply challenged offspring of control or nicotine fathers with a single dose injection of nicotine – these “naïve” animals had had no prior direct exposure to nicotine. In addition, we reasoned that since the animals in the self-administration paradigm were consuming nicotine for several weeks prior to eventual exposure to lethal levels of the drug (**Figure 2.6B**), this would be expected to substantially alter nicotine-related biology in the tested animal. We therefore also subjected TA and NIC offspring to one week of chronic low-dose nicotine (supplied in the drinking water) – we refer to these animals as the “chronic” cohort – then challenged these animals with an injection of a single LD_{50} dose of nicotine.

As shown in **Figure 2.7A**, naïve TA and NIC offspring exhibited no significant difference in susceptibility to a toxic nicotine injection, indicating that paternal nicotine exposure does not program a constitutively nicotine-resistant state. In contrast, and consistent with the results of the self-administration test, male (but not female) offspring of nicotine-exposed fathers became significantly more tolerant to a lethal nicotine challenge than control offspring (**Figure 2.7B**), but only once they had become acclimated to a week of chronic nicotine. Taken together, these data demonstrate that male offspring of nicotine-exposed fathers exhibit an enhanced ability to develop tolerance to toxic doses of nicotine, but that this tolerance is only revealed following prior exposure to sub-lethal levels of nicotine.

Figure 2.7:

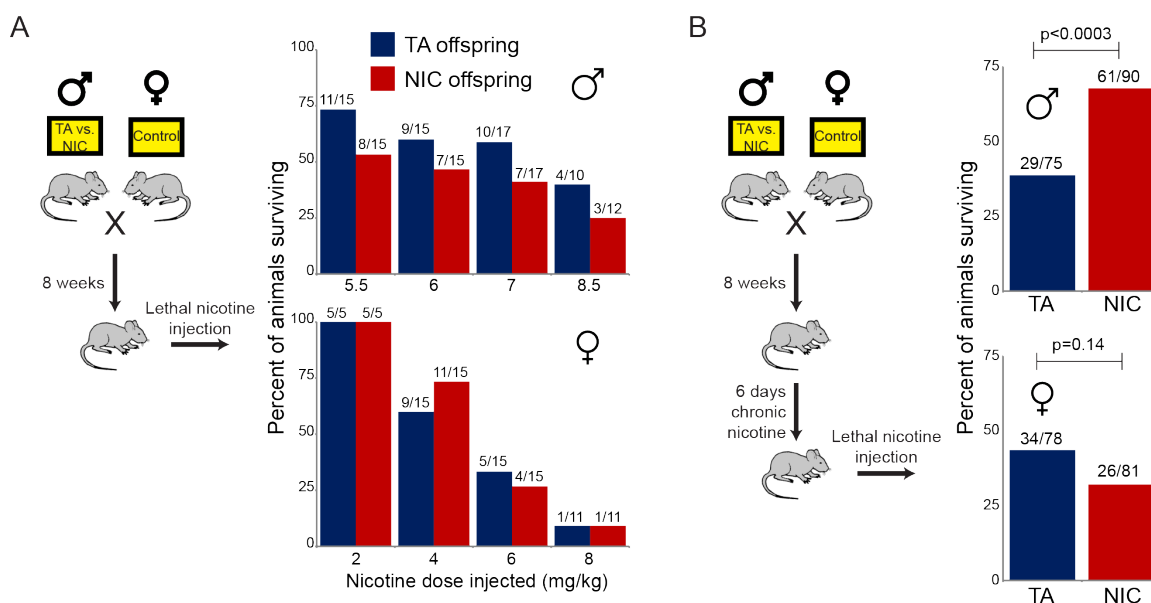


Figure 2.7. Paternally-induced protection from nicotine toxicity is primed by nicotine exposure in offspring.

(A) Survival of TA or NIC offspring following a single injection of nicotine at the indicated dose. Above each bar, fraction shows the number of surviving animals over number of animals injected. For all four doses tested, there was no significant difference in toxicity between TA and NIC offspring ($p > 0.7$ across all 4 doses for males, $p > 0.8$ for females).

(B) Survival of TA and NIC offspring following a single injection of nicotine at roughly the LD_{50} for naïve animals in (A) – 7.2 mg/kg for male offspring, shown in the top panel, 5.04 mg/kg for females, shown in the bottom panel. Here, offspring were acclimated to chronic nicotine in their drinking water for 6 days, with nicotine challenge being administered 24 hours following the last day of nicotine consumption.

Paternal nicotine exposure affects xenobiotic clearance in offspring

What is the physiological basis for the enhanced resistance to nicotine toxicity observed in NIC offspring relative to TA offspring? Lethal doses of nicotine induce seizures originating in the hippocampus (Fonck et al., 2003). Resistance to such seizures could result from highly specific resistance mechanisms such as downregulation of nicotinic acetylcholine receptors in the hippocampus, or from relatively nonspecific resistance mechanisms such as enhanced detoxification of xenobiotics in the liver. Although we cannot definitively rule out a neural basis for the enhanced nicotine resistance observed in NIC offspring, several lines of evidence – including extensive RNA-Seq analysis of

isolated hippocampus – argue against this resistance resulting from altered neural physiology (**Figure 2.8, File 2.1**).

Figure 2.8:

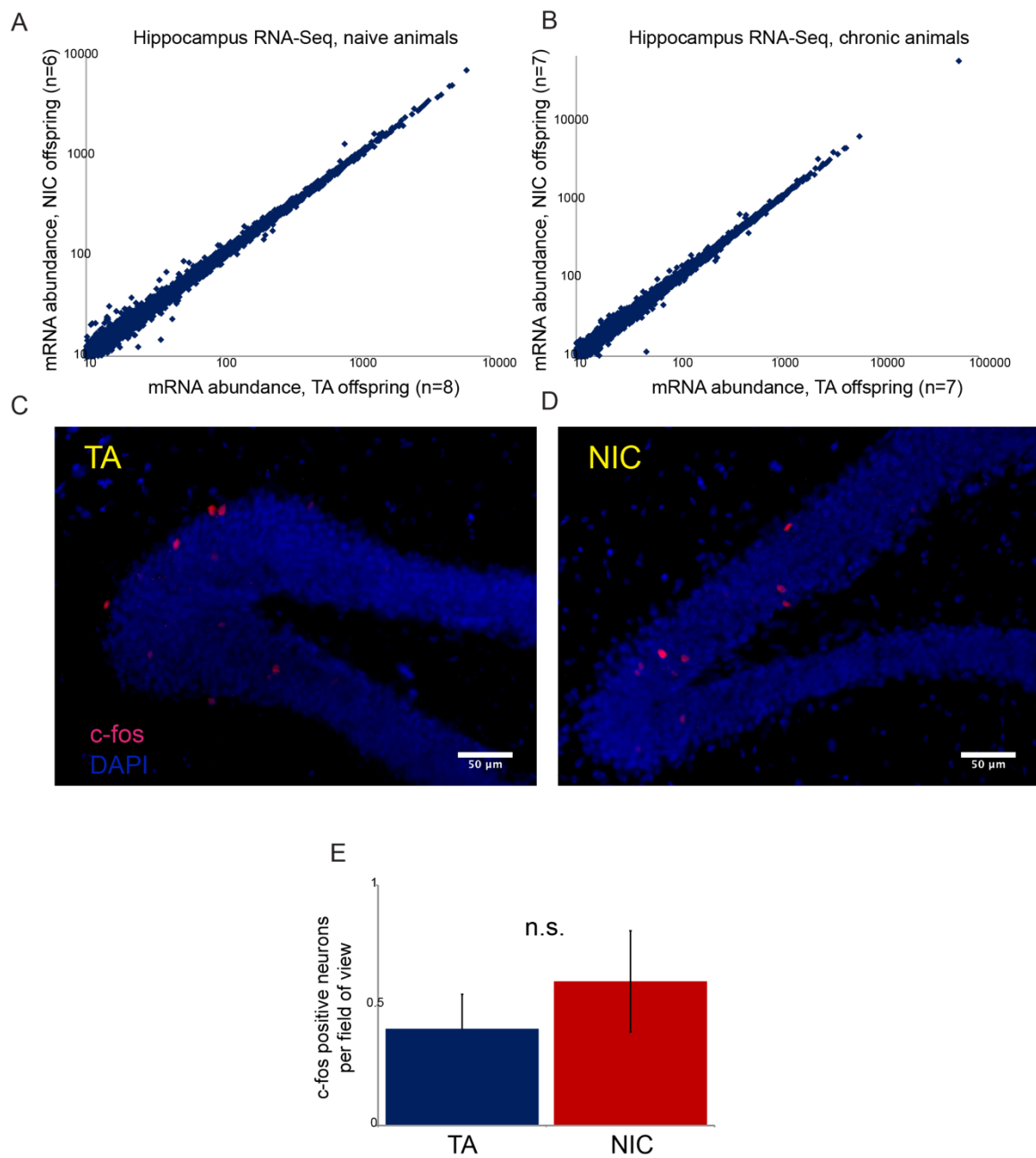


Figure 2.8. Paternal nicotine has no significant effects on offspring hippocampal gene regulation or neural activity.

(A-B) RNA-Seq of isolated hippocampus from TA and NIC offspring. Scatterplots show average mRNA abundance (minimum tpm of 10), with x axis showing average for TA offspring and y axis showing average for NIC offspring. (A) shows data for TA and NIC animals that had not experienced nicotine (“naïve”), (B) shows data for animals provided with chronic nicotine for 6 days. There are no significant effects of paternal nicotine exposure on any mRNAs in either condition. Similar results were obtained in preliminary studies of the ventral tegmental area, nucleus accumbens, and prefrontal cortex (not shown). Importantly, while recent reports document an increase in *Igf2* mRNA abundance in the hippocampus of 8 week old male offspring of stressed fathers (Short et al., 2016), we observed no significant change in *Igf2* levels in NIC offspring (see **File 2.1**), providing another argument against the hypothesis that our paternal nicotine exposure paradigm affects offspring via a paternal stress response.

(C-D) Paternal nicotine treatment does not affect offspring neural activity in the hippocampus. Representative images showing c-fos staining as a proxy for neural activity in hippocampus isolated from TA (C) and NIC (D) offspring. Here, animals were put on chronic nicotine (200 ug/ml nicotine free-base) for six days. After 24 hr without nicotine, animals were injected with 1.5 mg/kg nicotine free-base. Tissue was collected 90 min after the injection.

(E) Quantitation of c-fos staining data. Y axis shows number of c-fos-positive neurons in the gyrus dentatus for nicotine-injected TA (n=12), and NIC (n=13) offspring.

Several lines of evidence thus argue against the drug resistance of NIC offspring resulting from altered neural physiology. First, the fact that drug-acclimated animals exhibit enhanced resistance to both nicotine and cocaine toxicity (**Figures 2.7, 2.12**) rules out mechanisms involving downregulation or desensitization of either nicotinic acetylcholine receptors or the dopamine receptor. Second, RNA-Seq analysis of several brain regions – hippocampus, ventral tegmental area, nucleus accumbens, and prefrontal cortex -- revealed minimal effects of paternal nicotine exposure on the transcriptome. Finally, we found no significant differences in staining patterns of the activity marker c-fos in the hippocampus of TA and NIC offspring (**Figure 2.8C-E**). Thus, while we cannot definitively rule out a neural basis for the phenotypes observed in NIC offspring, we found no evidence to support such a hypothesis.

In contrast to the lack of relevant molecular changes observed in the brains of NIC offspring, we discovered a significant effect of paternal nicotine exposure on hepatic detoxification of nicotine in offspring. As shown in **Figure 2.9A**, nicotine-acclimated NIC offspring exhibit significantly higher levels of the long-lived nicotine metabolite cotinine at earlier time points after nicotine injection than do TA offspring. This finding is consistent with enhanced nicotine clearance underlying the nicotine resistance

phenotype displayed by these animals, suggesting that paternal nicotine exposure programs a state of enhanced metabolic tolerance in offspring.

Figure 2.9:

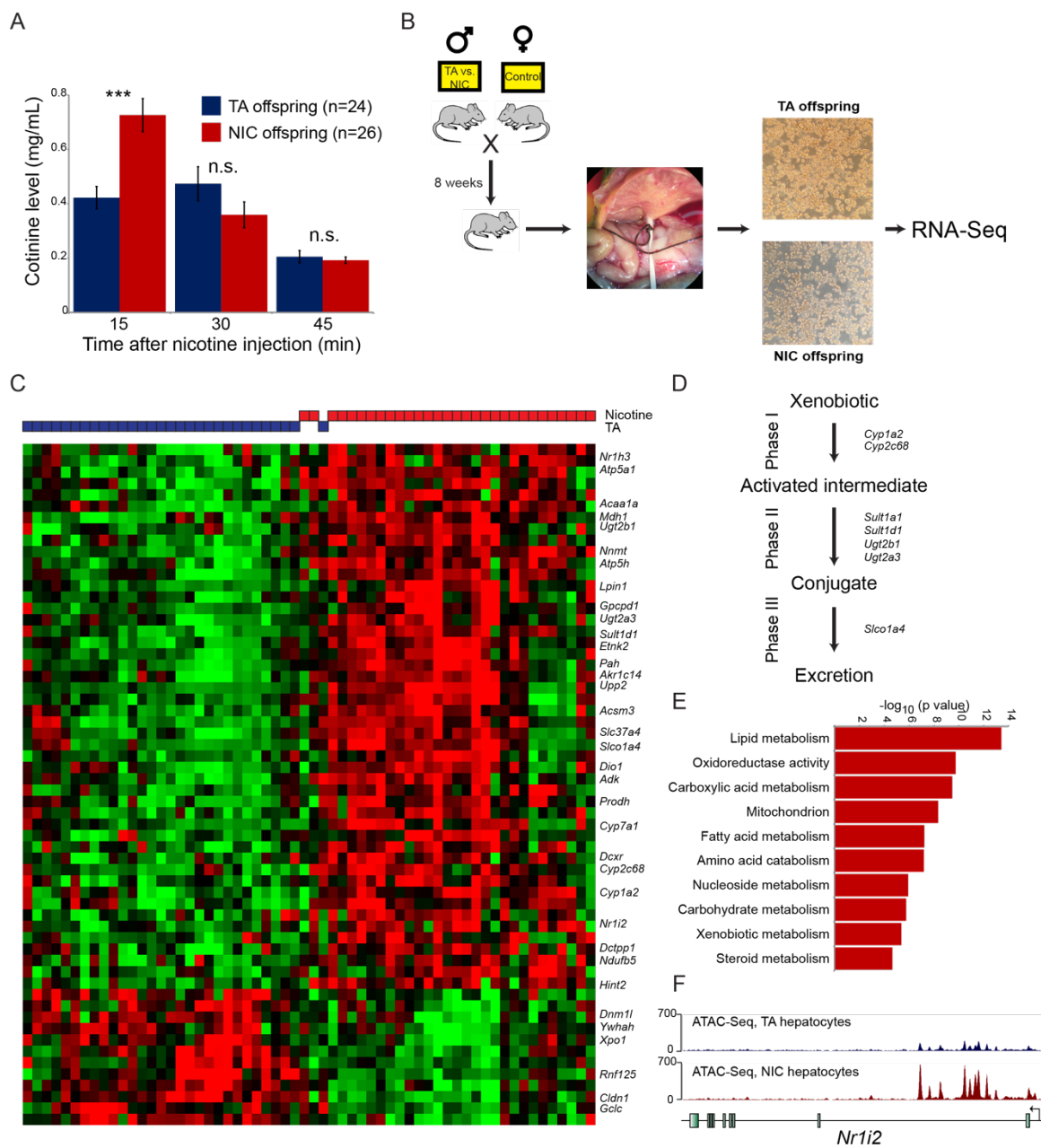


Figure 2.9. Paternal nicotine exposure induces an exaggerated protective response to xenobiotics.

(A) Paternal nicotine exposure enhances nicotine metabolism in offspring. Male TA and NIC offspring were acclimated to nicotine for 6 days, then 24 hours later were injected with 1.5 mg/kg nicotine. Serum levels of the long-lived nicotine metabolite cotinine were measured at the indicated times after nicotine injection, with significantly ($p < 0.0002$, t-test with Holm-Sidak correction) elevated cotinine levels being observed at the earliest time point analyzed, indicating enhanced nicotine clearance in NIC offspring.

(B) Schematic of hepatocyte RNA-Seq experiment.

(C) Cluster of hepatocyte RNA-Seq dataset. For each paternal treatment group (TA or NIC), data are shown for ten individual male offspring from ten separate litters, with hepatocytes from five animals also being cultured for varying times (0 to 21 hours) following isolation. Data are z score normalized for each culture time point. The heatmap shows 60 genes (filtered for average expression > 25 ppm) changing with a multiple hypothesis-corrected p value < 0.1 . Underlying data are provided in accompanying Source Data file.

(D) Genes upregulated in NIC offspring encode enzymes involved in all three phases of xenobiotic metabolism, as indicated.

(E) Selected Gene Ontology categories enriched among genes upregulated (adjusted $p < 0.1$) in NIC hepatocytes.

(F) ATAC-Seq coverage for TA and NIC hepatocytes, as indicated, across *Nr1i3*. See also **Figure 2.11**.

What is the molecular basis for the enhanced nicotine detoxification observed in NIC offspring? As the liver is the primary site of nicotine and other xenobiotic clearance in mammals, we investigated changes in mRNA abundance in hepatocytes isolated from TA and NIC offspring (**Figure 2.9B-C, File 2.2**). Paternal nicotine exposure significantly (adjusted $p < 0.05$) affected the expression levels of 51 genes, with upregulated genes being significantly enriched for those involved in lipid metabolism ($p=3.9e-14$), amino acid catabolism ($p=6.6e-8$), and various mitochondrial annotations including mitochondrial membrane ($p=1.9e-7$) (**Figure 2.9D-E, Figure 2.10A-B**). Most notably, given the nicotine resistance observed at the organismal level, NIC hepatocytes also exhibited increased expression of genes involved in drug metabolism ($p=4.3e-6$), with upregulated genes including “Phase I” (*Cyp1a2*, *Cyp2c68*) and “Phase II” (*Ugt2a3*, *Ugt2b1*, *Sult1d1*, and *Sult1a1*) detoxification enzymes, “Phase III” membrane transporters (*Slco1a4*), as well as genes encoding the xenobiotic-responsive nuclear hormone receptors CAR and PXR (*Nr1h3* and *Nr1i2*) (**Figure 2.9C-D**). In addition, the primary cytochrome involved in nicotine clearance in rodents, *Cyp2a5*, was upregulated ~2-fold on average in NIC hepatocytes. Although this up-regulation was not significant (adjusted $p=0.2$) in the genome-wide dataset due to sample to sample variability in expression of this gene, we validated up-regulation of *Cyp2a5* in additional intact livers (n=6 NIC, n=4 TA, $p<0.01$) by q-RT-PCR (**Figure 2.10C**).

Figure 2.10:

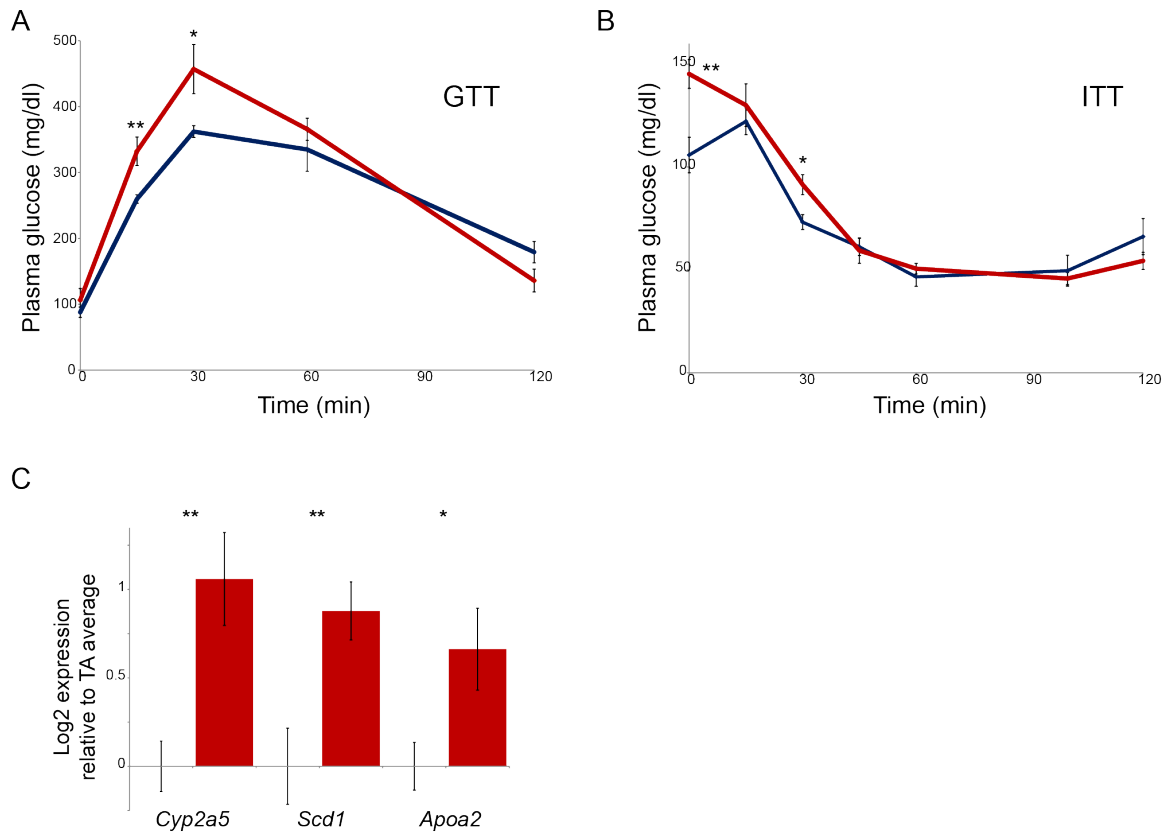


Figure 2.10. Paternal nicotine exposure affects multiple phenotypes in offspring.

(A-B) Glucose tolerance (A) and insulin tolerance (B) are significantly altered in NIC offspring relative to TA offspring. Plasma glucose levels are shown for 6 male NIC or TA offspring at varying times after a 2 g/kg glucose bolus at 7 weeks of age (A), or a 0.75 U/kg insulin bolus at 10 weeks of age (B). * and ** represent p values of <0.05 and <0.01, respectively (t-test).

(C) q-RT-PCR data for the indicated genes. In each case, expression level (after normalizing to *Actb* and *Gapdh*) is plotted relative to the average expression level for 4 TA livers (n=6 NIC livers), with bars showing average and S.E.M.

These gene expression studies thus reveal that, relative to TA hepatocytes, NIC hepatocytes exhibit a general derepression of target genes for a broad range of nuclear hormone receptors. To investigate the mechanistic basis for this derepression, we characterized open chromatin genome-wide in TA and NIC hepatocytes (n=8 samples each) using ATAC-Seq (Buenrostro et al., 2015). Our ATAC-Seq dataset exhibited expected features such as strong peaks of accessibility over promoters and other regulatory elements (**Figure 2.11**). Comparing TA and NIC datasets, we observed a consistent global difference in overall chromatin accessibility – normalized ATAC peaks at regulatory elements were nearly 2-fold higher in NIC hepatocytes than in TA hepatocytes, while TA hepatocytes exhibited a consistently higher background of transposition throughout regions of the genome distant from regulatory elements (**Figure 2.11A-C**). Whatever the basis for this global change in chromatin accessibility, we additionally identified 1861 peaks of chromatin accessibility (**Figure 2.9F**, **Figure 2.11D-H**, **File 2.3**) that differ significantly between TA and NIC hepatocytes after correcting for the global difference in peak height between these samples. Consistent with the changes in mRNA abundance observed in hepatocytes, these peaks were significantly enriched near genes involved in lipid metabolism ($p=2.8e-18$) and xenobiotic metabolism ($p=1.3e-6$), along with many related GO categories. We conclude

that a history of paternal drug exposure can influence the chromatin landscape of hepatocytes in offspring, resulting in a broad increase in accessibility at regulatory elements involved in metabolism and detoxification.

Figure 2.11:

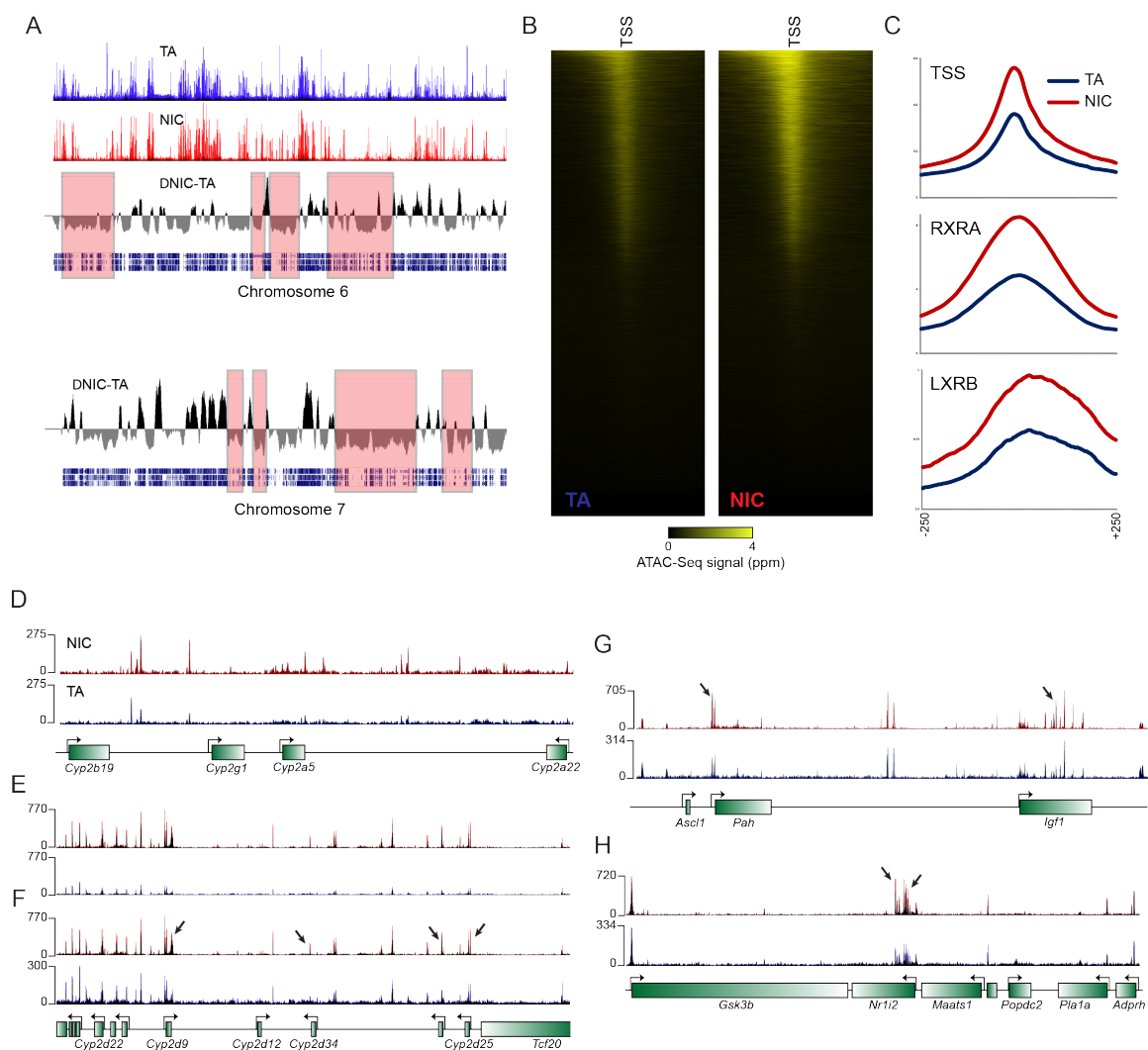


Figure 2.11. Global differences in hepatocyte chromatin architecture between TA and NIC offspring.

Aggregated ATAC-Seq data for hepatocytes isolated from TA and NIC offspring (n=4 animals each, with dexamethasone-treated and -untreated samples for each animal).

(A) Multimegabase-scale differences in the accessibility landscape of TA and NIC hepatocytes. Top two panels show ATAC-Seq data for NIC and TA offspring for chromosome 6, along with an averaged NIC-TA score, followed by gene density. For chromosome 7, only NIC-TA and gene density are shown. Red boxes highlight a subset of genomic regions of low gene density in which TA hepatocytes exhibit greater ATAC-Seq signal than NIC hepatocytes. Conversely, gene-dense regions generally exhibit higher ATAC signal in NIC hepatocytes (not highlighted). The mechanistic basis for this global difference is unclear – it does not appear to reflect contamination of TA samples with dead cells, for example, as these samples (from nicotine-naïve animals) did not differ in viability, and plating of hepatocytes also effectively selects against dead cells. As fragment length distributions were consistent from library to library, it also seems unlikely that there were gross differences in the concentration or activity of the added Transposase Tn5. Nonetheless, while this difference could reflect meaningful biology, such as a global difference in heterochromatin condensation, global differences in any genome-wide assay should of course be viewed with skepticism.

(B) ATAC-Seq data for 500 bp surrounding all annotated transcription start sites (TSSs), sorted from high to low average ATAC signal intensity.

(C) Increased ATAC signal in NIC hepatocytes is shown for all TSSs, or RXRA or LXRB binding sites, as indicated.

(D-H) Examples of loci exhibiting enhanced chromatin accessibility in NIC offspring, relative to TA offspring. (D-E) show ATAC-Seq tracks in which TA and NIC data are set to the same vertical range, as in **Figure 2.9F**. For panels F-H, y axes are set independently for TA and NIC datasets, visually correcting for the global differences between TA and NIC datasets. In these panels, a subset of significantly NIC-enriched peaks (**File 2.3**) are indicated with arrows.

Enhanced xenobiotic resistance in NIC offspring is not specific for nicotine

Importantly, the gene expression program observed in isolated hepatocytes includes a broad variety of genes associated with drug metabolism, most of which are not specific for nicotine clearance. To test the hypothesis that the nicotine-resistant state of NIC offspring reflects a general xenobiotic response, rather than a nicotine-specific detoxification pathway, we asked whether NIC offspring also exhibit enhanced resistance to another toxic challenge, cocaine. As cocaine and nicotine operate through distinct molecular pathways – cocaine prevents dopamine reuptake at the synaptic cleft by binding to and blocking the dopamine transporter, while nicotine activates and desensitizes nicotinic acetylcholine receptors – a finding of enhanced tolerance to cocaine would strongly argue against NIC offspring exhibiting specific epigenetic effects on the direct molecular receptor for nicotine.

We first assessed cocaine toxicity in “naïve” animals that had not been previously directly exposed to nicotine or cocaine. Similar to our findings with nicotine toxicity (**Figure 2.7A**), naïve NIC and TA animals did not exhibit significant differences in their resistance to cocaine toxicity (**Figure 2.12A**). However, as the enhanced ability of NIC offspring to survive toxic nicotine levels was only revealed following pre-exposure of these animals to sub-lethal doses of nicotine (**Figure 2.7B**), we next sought to determine whether acclimation of NIC offspring to cocaine could induce a cocaine-resistant state. To address this question, TA and NIC offspring were chronically treated with sub-lethal doses of cocaine – twice-daily injections of 15 mg/kg cocaine for five days – prior to challenge with a toxic dose of cocaine. Astonishingly, this acclimation protocol resulted in enhanced resistance to cocaine toxicity in NIC offspring, relative to TA controls (**Figure 2.12B**), revealing that NIC offspring are hyper-responsive to multiple xenobiotics.

Figure 2.12:

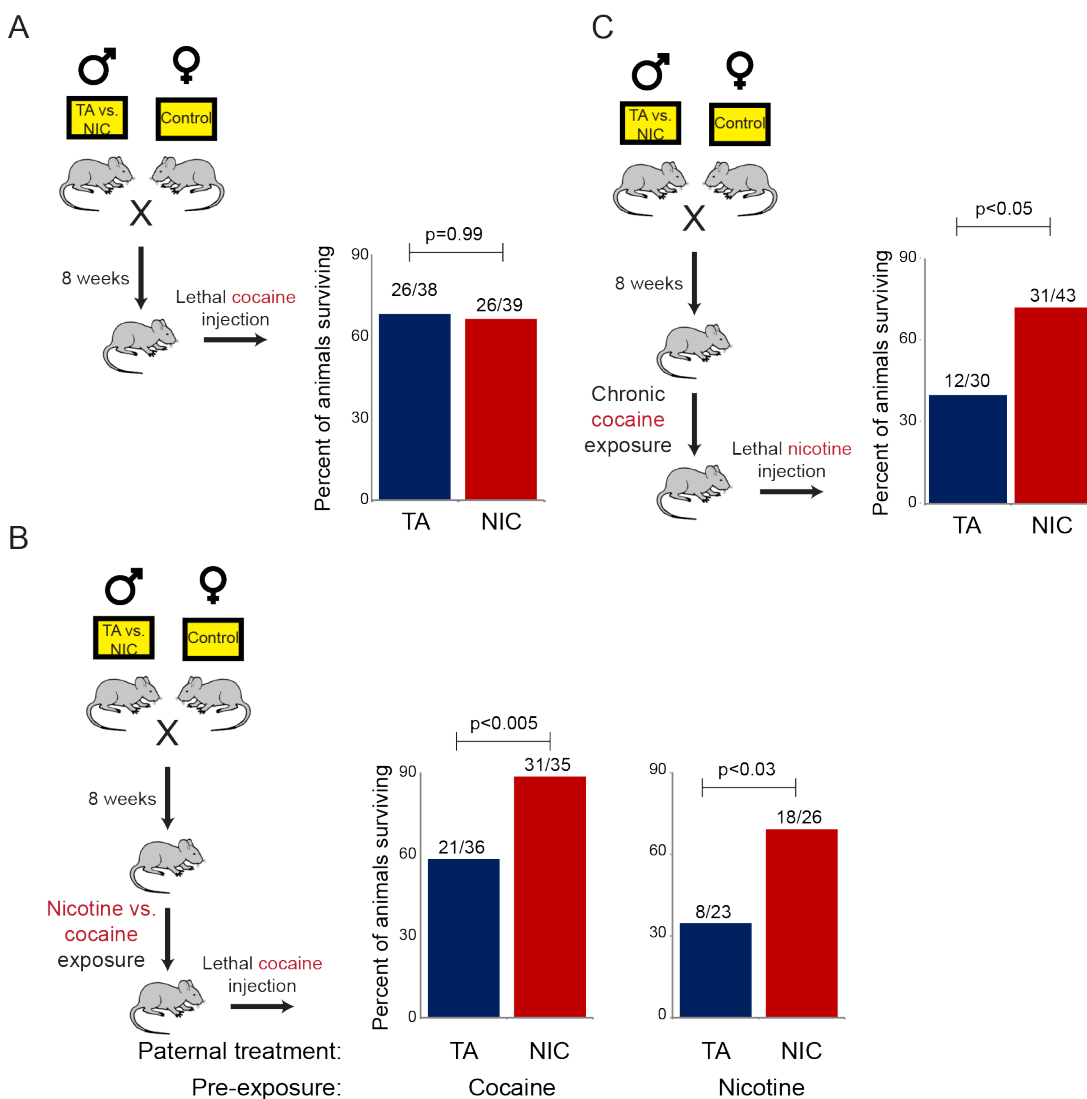


Figure 2.12. NIC offspring are protected from multiple xenobiotics.

(A) Paternal nicotine exposure does not affect susceptibility of drug-naïve offspring to cocaine toxicity. Male TA and NIC offspring were injected with a single 100 mg/kg dose of cocaine. Survival is shown as in Figure 2.7.

(B) Acclimation of TA and NIC offspring to either nicotine or to cocaine reveals protective effect of paternal nicotine exposure on offspring cocaine resistance. As in (A), for male offspring acclimated to chronic nicotine (200 µg/mL nicotine free-base in drinking water for six days) or cocaine (twice-daily injections with 15 mg/kg cocaine for five days). Twenty-four hours following final drug exposure, animals were injected with a single 100 mg/kg dose of cocaine.

(C) Cocaine acclimation induces nicotine resistance in NIC offspring. Here, male TA and NIC offspring were acclimated to cocaine injections (twice-daily, 15 mg/kg) over five days. Twenty-four hours after the final cocaine injection, animals were injected with 7.2 mg/kg nicotine.

We next asked whether the process of acclimation to sub-lethal doses of nicotine or cocaine induces a drug-specific resistant state in NIC offspring. In other words, does pre-acclimation of NIC offspring to different molecules induce resistance specifically to the drug to which the animals were exposed, or do chronic exposures to multiple distinct drugs all induce a common state of general xenobiotic resistance? To distinguish these possibilities, we pre-acclimated TA and NIC offspring to either nicotine or cocaine, then challenged acclimated animals with a lethal dose of the drug to which they had not yet been exposed. Consistent with the hypothesis that drug acclimation induces a general xenobiotic response, we found that pre-acclimation to nicotine induced a cocaine-resistant phenotype in NIC offspring, and, conversely, that chronic cocaine could induce

nicotine resistance (**Figures 2.12B-C**). Together, these data suggest that paternal nicotine exposure programs a hyper-responsive state in male offspring in which chronic xenobiotic exposure results in a generalized toxin resistance.

Drug resistance is induced by multiple paternal drug exposures.

The revelation that nicotine resistance in NIC offspring reflects a somewhat generic xenobiotic resistance program (**Figures 2.9C-D, 12**) raises the question of what aspect of the paternal nicotine exposure paradigm is responsible for programming the offspring phenotype. The nicotine exposure paradigm utilized here induces nicotinic acetylcholine receptor (nAChR) signaling, with several physiological consequences: 1) nicotine dependence, 2) reduced caloric intake, and 3) physiological withdrawal resulting from the removal of nicotine for the final week prior to mating. To investigate the role of nAChR signaling in the paternal induction of offspring drug resistance, we made use of mecamylamine, a non-selective, non-competitive antagonist of nAChRs that readily crosses the blood-brain barrier.

Male mice were provided with 2.0 mg/kg/day mecamylamine via a surgically-implanted infusion pump, and mecamylamine-treated mice were split to either nicotine or TA drinking water, as in our primary nicotine exposure paradigm. Studies have previously shown that mecamylamine administration prevents known physiological responses to nicotine such as nicotine-induced anorexia (Mineur et al., 2011), hypothermia and locomotor effects (Tapper et al., 2004), and nicotine reinforcement (Corrigall and Coen,

1989). Male offspring of these fathers were then acclimated to nicotine for 6 days, then subject to a toxic nicotine challenge, as in **Figures 2.7 and 2.12**. Surprisingly, male mice concurrently treated with nicotine and its antagonist fathered offspring with the same enhanced nicotine resistance seen in NIC offspring (**Figure 2.6**). Importantly, this finding rigorously rules out the possibility that our nicotine exposure paradigm induces paternal effects on offspring as a consequence of the nicotine withdrawal stress imposed in the week before mating.

Moreover, the drug resistance observed in nicotine + mecamylamine offspring strongly argues that this paternal effect does not even require nicotine signaling in treated fathers, instead suggesting that the paternal effect is perhaps induced simply by exposure to xenobiotics. Consistent with this hypothesis, mecamylamine exposure alone also induced drug resistance in the next generation, although this effect was not as robust as that induced by nicotine or nicotine + mecamylamine (**Figure 2.13**). Together, these data demonstrate that drug resistance in sons can be induced by paternal exposure to both nAChR agonists and nAChR antagonists, arguing that paternal xenobiotic exposure is likely to be the relevant feature of our nicotine exposure paradigm.

Figure 2.13:

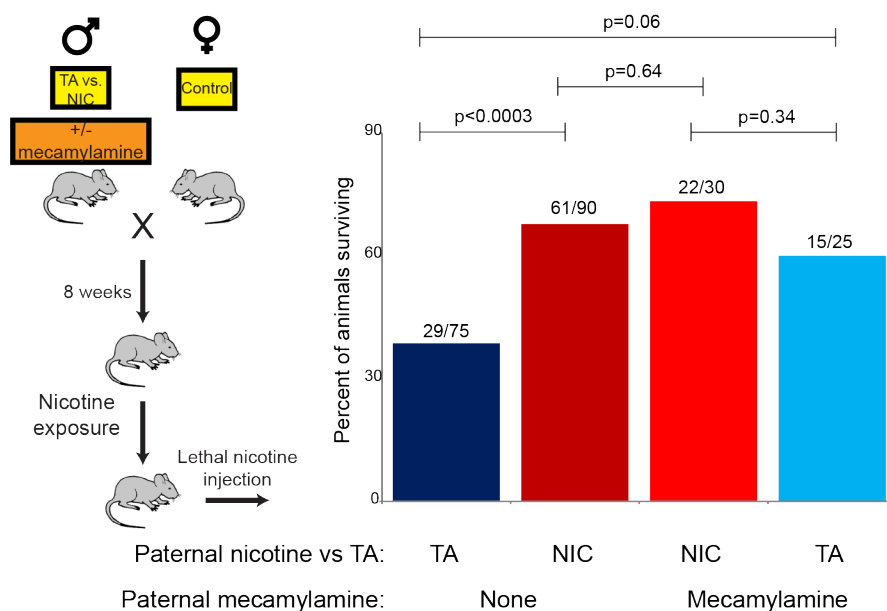


Figure 2.13. Offspring drug resistance is induced by a nicotine antagonist.

Here, we modified the paternal exposure paradigm by implanting pumps to deliver the nicotine antagonist mecamlamine to male mice. Mecamlamine-treated mice were provided with nicotine or control solution for four weeks, then mated to control females. Male offspring were acclimated to chronic nicotine for six days and then subject to a toxic nicotine challenge, and survival is shown as in **Figures 2.3, 2.5**. Data for no mecamlamine animals are reproduced from **Figure 2.3B**. Note that concurrent mecamlamine and nicotine exposure resulted in a protective effect on offspring, and even mecamlamine alone was able to modestly induce nicotine resistance in the next generation.

Relative sparing of hepatocytes following drug treatments in NIC offspring.

Finally, we sought to understand the requirement for drug acclimation in revealing organismal drug resistance in NIC offspring. Curiously, the relative up-regulation of xenobiotic processing genes (XPGs) in NIC offspring was observed in hepatocytes and livers isolated from “naïve” animals that had not been exposed to nicotine or cocaine (**Figure 2.9**), yet enhanced resistance to toxins was only observed in animals that were first acclimated to one of these drugs (**Figures 2.7, 2.12**). To test the hypothesis that XPG up-regulation might be even stronger in NIC hepatocytes following drug exposure, we set out to characterize gene expression changes in nicotine- or cocaine-acclimated offspring. However, in attempting to isolate hepatocytes from drug-acclimated TA and NIC offspring for RNA-Seq analysis, we noticed much poorer recovery of hepatocytes from TA than from NIC offspring (not shown), suggesting the possibility that NIC animals might be protected from drug-induced hepatotoxicity. Therefore, to quantify cell viability in vivo, we took a histochemical approach to assess apoptosis in livers from drug-acclimated TA and NIC offspring. Consistent with the relatively poor recovery of hepatocytes from TA animals, we observed substantial hepatocyte death in the livers of cocaine-exposed animals (**Figure 2.14A**). Importantly, while hepatocyte apoptosis and necrosis were extremely common in livers from cocaine-exposed TA offspring, NIC offspring were significantly protected from such cocaine toxicity (**Figure 2.14**). We conclude that the up-regulation of XPGs in naïve NIC offspring is not sufficient to significantly protect animals from a lethal nicotine or cocaine challenge, but that this up-regulation can protect hepatocytes from sub-lethal doses of these drugs. Following a

week of chronic toxin exposure, TA offspring are left with substantially reduced liver function, while NIC offspring maintain greater numbers of functional hepatocytes. We speculate that this greater hepatocyte functional capacity, as well as the up-regulation of XPGs in hepatocytes (**Figure 2.9**), may both serve to protect the animal from a single toxic dose of xenobiotic.

Figure 2.14:

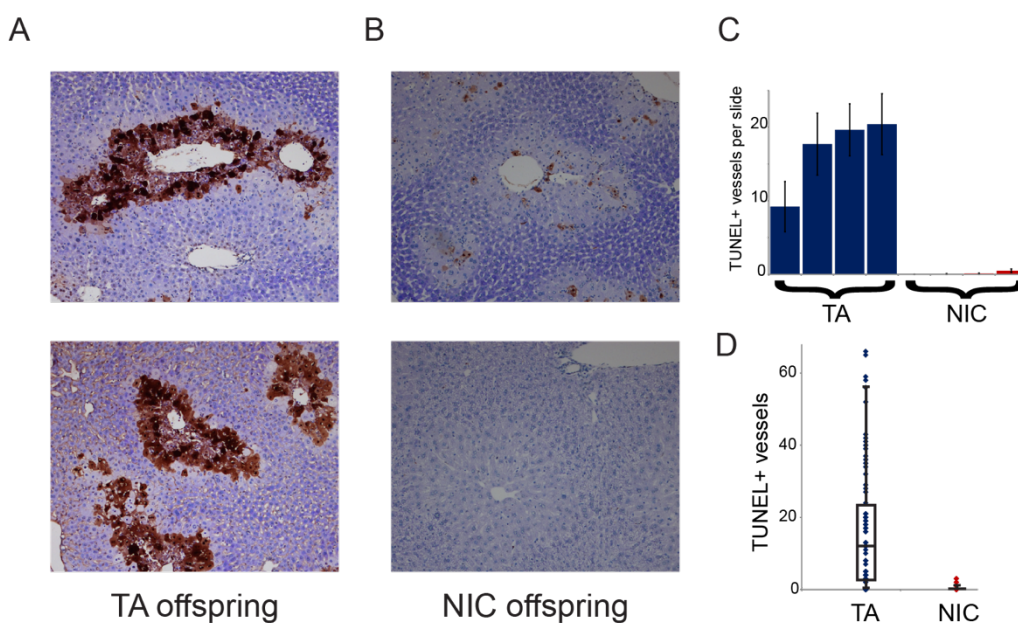


Figure 2.14. NIC offspring exhibit relative sparing of hepatocytes following chronic drug exposure.

(A-B) Effects of chronic cocaine treatment on hepatocyte viability. Two representative sections are shown for TUNEL-stained livers from TA (A) and NIC (B) offspring following 5 days of cocaine injections (twice-daily, 15 mg/kg). Prominent centrilobular apoptosis is seen in TA offspring, but is almost completely absent in NIC offspring.

(C-D) Quantitation of TUNEL staining data. (C) shows the average (plus/minus s.e.m.) number of TUNEL+ centrilobular regions per slide (staining of >25% of central vein circumference was counted as TUNEL+, and was assessed at five different levels for each liver lobe I-IV) for 4 individual TA (blue) and NIC (red) offspring, treated as in (A-B). (D) shows data for all individual slides as dots, with boxplot showing median, 1 standard deviation, and 5th/95th percentile for the 80 data points.

Discussion

Here, we report a novel paradigm for intergenerational effects of paternal environment on offspring phenotype, based on paternal nicotine administration. Our data reveal that paternal nicotine exposure programs a state of nicotine resistance in offspring, but, surprisingly, neither the paternal sensing machinery nor the offspring response are specific for nicotine.

Paternal nicotine exposure induces a pleiotropic, nonspecific set of phenotypes in offspring.

The use of nicotine, a well-characterized small molecule that acts in vivo by binding to specific receptors, as the inciting paternal exposure enabled us to rigorously interrogate the specificity of the offspring response. Importantly, the enhanced toxin survival seen in offspring is not specific for the drug to which fathers were exposed – NIC offspring were hyper-resistant to both nicotine and to cocaine challenges – demonstrating that our paternal exposure paradigm does not result in transmission of a nicotine-specific phenotype to progeny (at least for toxicity, locomotor effects, and reward behavior).

Mechanistically, the drug resistance observed in NIC offspring presumably results from the enhanced hepatic drug clearance observed in these animals (**Figure 2.9A**). Consistent with this increased nicotine clearance, isolated hepatocytes exhibited up-regulation of a variety of xenobiotic processing genes (XPGs) accompanied by greater chromatin accessibility at relevant regulatory regions. A variety of XPGs are induced in NIC

hepatocytes in addition to those known to play a role in nicotine clearance (**Figure 2.9C**), suggesting that NIC offspring may prove resistant to many toxins beyond the two tested in this study.

In addition to the significant de-repression of xenobiotic response genes observed in NIC offspring, we note that the most significant effects of paternal nicotine on offspring hepatocyte gene expression occurred at metabolic genes (**Figure 2.9C, E**). This finding suggested that NIC offspring might also exhibit metabolic alterations, in addition to the documented changes in xenobiotic resistance. Alterations in glucose control and lipid metabolism are commonly observed in paternal effect studies, being observed not only in dietary paradigms, but also in some stress and toxin-related paternal effect studies (Rando and Simmons, 2015), suggesting that multiple distinct stimuli experienced by males might in some way convergently influence metabolic traits in offspring. As a detailed metabolic phenotyping of NIC offspring is beyond the scope of this study, we chose here to simply focus on the most common phenotype observed in other paternal effect experiments, assaying glucose and insulin tolerance in TA and NIC offspring (**Figure 2.10**). Consistent with the ability of multiple paternal environments to alter glucose control in offspring, we observed that NIC offspring exhibited significantly diminished clearance of a glucose bolus, as well as a moderately diminished response to insulin.

Taken together, our data reveal 1) that paternal nicotine exposure induces a pleiotropic set of phenotypes in male offspring, and 2) that the induced phenotypes in offspring are not specific for nicotine. It will be of great interest in future studies to interrogate a wide variety of phenotypes in offspring of males subject to a broad range of exposure

paradigms – including stress, nicotine treatment, and various diets – to identify common and divergent phenotypes induced by distinct paternal exposure paradigms.

Paternal programming of offspring drug resistance is limited to male offspring.

A curious feature of many, but not all, paternal effect paradigms reported in mammals is that phenotypic effects often manifest preferentially in offspring of one gender. For example, while paternal social defeat was reported to affect anxiety-related behavior in both male and female offspring, locomotor activity and sucrose preference were only altered in male offspring (Dietz et al., 2011). Here, we find that paternal nicotine exposure only affects drug resistance in male offspring, raising once again the unsolved question of why paternal environments induce gender-specific outcomes in progeny. Here, we consider three potential explanations for this phenomenon.

First, a subset of epigenetic information carriers – cytosine methylation and chromatin packaging – are associated in cis with a specific genomic locus, meaning that epigenetic changes occurring on the sex chromosomes will only affect progeny inheriting that chromosome. Thus, it is plausible that nicotine exposure affects epigenetic modification of the Y chromosome to program drug resistance in male offspring (or, less simply, that epigenetic marks on the X chromosome suppress an autosomal or small RNA-directed phenotype that would otherwise affect both male and female progeny). Second, X chromosome dosage compensation in mammals occurs via silencing of one of the two X chromosomes in females. The inactive X chromosome could thus act as a “sink” for

epigenetic silencing machinery in females (Blewitt et al., 2005), such that the effective levels of this machinery available for autosomal gene regulation could differ between males and females. In this scenario, paternal transmission of an epigenetically-marked autosomal locus, or RNA, could cause differential effects in developing male vs. female offspring based on differences in the available levels of epigenetic effector machinery. Finally, we note that an emerging theme in many paternal effect paradigms is that the phenotypic changes observed in offspring are known to be regulated by various nuclear hormone receptors (NHRs). For example, the phenotypes described in paternal stress paradigms are related to glucocorticoid receptor signaling, while the metabolic gene expression changes resulting from paternal dietary interventions exhibit significant overlap with genes regulated by NHRs such as PPAR α (Carone et al., 2010). Here, we find that paternal nicotine exposure affects hepatic expression of many targets of metabolic NHRs, as well as the xenobiotic-responsive NHRs CAR and PXR (**Figure 2.9**). As sex hormones also act through NHR signaling – androgen receptor and estrogen receptor – we speculate that levels or activity of NHR coactivators or corepressors could differ in male vs. female progeny, resulting in altered penetrance or magnitude of paternal effects on NHR-mediated gene regulation.

Offspring drug resistance is revealed by pre-exposure to xenobiotics.

A crucial feature of the drug resistance exhibited by NIC offspring is that the toxin-resistant state is only revealed by pre-exposure of these animals to xenobiotics. This requirement for drug pre-exposure/acclimation emphasizes the key role of the *offspring's* environment in the manifestation of an epigenetically “reprogrammed” phenotype. In other words, the development of an animal’s phenotype here involves an interaction between environmental conditions in two consecutive generations (see (Rodgers et al., 2013; Zeybel et al., 2012) for similar examples) – as with gene X environment effects, epigenetic marks also have context-dependent effects on organismal phenotype.

What is the mechanism, by which low level drug exposure enhances the survival of NIC offspring? NIC hepatocytes exhibit derepression of xenobiotic response genes even before exposure to any drugs, yet these drug-naïve animals are no more resistant to nicotine or cocaine toxicity than control animals (**Figures 2.7A, 2.12A**). Instead, the enhanced xenobiotic metabolism in NIC livers appears to protect susceptible hepatocytes from toxicity during a course of sub-lethal drug exposure (**Figure 2.14**). The loss of hepatocytes in drug-exposed TA animals presumably explains why fewer than 50% of these animals survive an LD50 dose – calculated using drug-naïve animals – of nicotine or cocaine (**Figures 2.7, 2.12**), with the preservation of hepatic capacity in NIC offspring preventing this degradation in survivability. That said, not only do drug-acclimated NIC offspring simply *preserve* their survival in the face of an LD50 dose of these drugs, but they exhibit dramatically *improved* survival, as far more than half of these animals survive this challenge. We have yet to uncover the mechanistic basis for this enhanced

survival, as RNA-Seq analysis of the hepatocytes isolated from drug-acclimated animals does not reveal further up-regulation of XPGs than that documented for naïve hepatocytes (not shown). Future studies will investigate whether drug acclimation might

1) affect mRNA abundance in a limited subset of hepatocytes (which would be diluted out in whole liver or hepatocyte culture experiments),

2) affect mRNA abundance only transiently during drug exposure (and not in cultured hepatocytes), leaving behind higher levels of the encoded proteins without an mRNA-Seq signature, or

3) affect xenobiotic metabolism not at the level of mRNA abundance, but post-transcriptionally.

How is nicotine sensed in exposed males?

The pleiotropic response observed in nicotine-exposed offspring raises the question of how nicotine is sensed in the paternal generation in this system. A key question in this regard is whether stress experienced by the nicotine-exposed males might be responsible for inducing the offspring phenotype, as it is known that a variety of paternal stress exposure paradigms – including early maternal separation, social defeat stress, and chronic variable low level stress – affect multiple phenotypes in offspring, from glucose control to anxiety-related behaviors (Bale, 2015). While we have not formally ruled out a role for paternal stress in our system – it will of course be of interest to assay offspring

nicotine resistance in well-studied paternal stress paradigms – two findings strongly argue against this paternal effect arising from a general stress response. First, chronic exposure to the nicotinic receptor antagonist mecamylamine, which blocks nicotine dependence in nicotine-treated fathers, does not interfere with induction of xenobiotic resistance in offspring (**Figure 2.13**), thus definitively ruling out a role for paternal withdrawal stress in induction of this phenotype. This first point is further supported by the finding that mecamylamine alone – which on its own has little effect on anxiety, locomotor behavior, or physical withdrawal symptoms in nicotine-naïve mice (Zhao-Shea et al., 2013) – is sufficient to induce xenobiotic resistance in offspring. Second, in contrast to multiple reported paternal stress paradigms, we do not find any evidence that paternal nicotine exposure affects anxiety-related behavior in offspring (**Figure 2.2**).

What, then, is the relevant feature of nicotine in inducing xenobiotic resistance in offspring? Paternal effects on toxin resistance in offspring did not require nicotinic receptor signaling, as both nicotine itself as well as a nicotine antagonist were able to induce the protective response in offspring. As both nicotine and mecamylamine exposure can result in reduction of nAChR signaling via desensitization or antagonism, respectively, it is formally possible that nAChR deactivation is the inciting stimulus in the paternal generation (or, less likely, that the surgical stress of mecamylamine infusion pump implantation, and nicotine consumption, both convergently induce the same effect in offspring). However, we favor the simpler hypothesis that both of these molecules serve to program offspring drug resistance via effects on paternal xenobiotic sensing. This model naturally raises the question of how xenobiotic exposure is sensed. As a

diverse variety of xenobiotics can affect gene regulation via activation of the NHRs CAR and PXR, these NHRs represent appealing candidates for the relevant xenobiotic sensor in fathers.

Whatever the nature of the relevant xenobiotic sensor, a key challenge to address is why experimental exposure to nicotine or mecamylamine (or, presumably, many other xenobiotics) reprograms offspring drug resistance relative to control animals, given that control animals are also exposed to a multitude of small molecules even in controlled laboratory conditions. Do nicotine and mecamylamine somehow induce a switch-like “all or none” change in some epigenetic mark that is not present in control sperm, or is the overall activity level of a xenobiotic sensor translated into quantitative changes in the levels of some continuous signal present in sperm? In the former case, what aspects of a given exposure paradigm are required to induce alterations to the sperm epigenome? We offer that one appealing mechanism for sensing increased levels of environmental xenobiotics would rely on comparing changes in sensor activity over an animal’s lifetime. For instance, if CAR/PXR signaling early in life – in utero perhaps, or early in postnatal life – were to result in a long-lasting “setpoint” for the levels of CAR/PXR activity expected later in life, then the organism could detect increased xenobiotic exposure later in life via changes in overall CAR/PXR activity compared to this setpoint. Future studies will explore the nature of the “nicotine” sensor in the paternal generation, and how information about exposure history is transmitted to offspring.

Taken together, our studies define a novel paternal exposure paradigm based on a specific ligand-receptor interaction, and show that paternal nicotine exposure programs offspring for enhanced resistance to multiple distinct toxins. Our data also reveal broad metabolic gene expression changes in NIC offspring, with potential implications for metabolic and cardiovascular health of offspring. Future studies will determine whether paternal nicotine exposure affects offspring via epigenetic marks in the sperm (vs. seminal fluid, etc.), and how paternally-transmitted information alters the course of development to result in xenobiotic-resistant hepatocytes. It will also be of interest to extend these studies to human populations, where the longer half-life of nicotine could potentially result in self-administration phenotypes not observed in the mouse model.

Acknowledgement

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Materials and Methods

Animal husbandry and drug treatments: C57BL/6J mice (RRID:IMSR_JAX:000664), 3 weeks old, were obtained from Jackson labs on a weekly basis and group-housed (4 mice/cage) on a 12-hr light-dark cycle (7:00 A.M. to 7:00 P.M). After arrival, males were immediately put on either tartaric acid (TA, 375 µg/ml) or nicotine (200 µg/ml nicotine free-base) in drinking water for 5 consecutive weeks, followed by an additional week on tap water prior to mating. Nicotine-exposed and control males were then allowed to mate (for six days) with control females from the same shipment date. F1 offspring from nicotine-exposed and control fathers were used for all experiments reported, generally at 8 weeks of age unless otherwise noted. Animals were maintained on-site in accordance with an approved IACUC protocol (A-1788).

Locomotor assay: F1 males from nicotine-exposed and control fathers were pre-conditioned to handling and injections with 0.9% saline (100 µl, i.p.) for three days prior to start of the study. For the nicotine test sessions, animals were injected with nicotine and transferred to individual cages placed within an infrared photobeam frame (San Diego Instruments). Test sessions lasted 40 or 90 min per day for 9 consecutive days. Locomotor activity was defined as the number of beam breaks during a session, whereupon the animal had to cross at least 2 photobeams from the original location to

count as ambulation. Results were statistically quantified using unpaired t-tests with multiple comparison adjustment (Holm-Sidak correction).

Nicotine Self-Administration Assay: Microsurgical catheter implant was performed on 7-week old F1 males from nicotine-exposed and control fathers. Animals were anaesthetized with ketamine (100 mg/kg BW) and xylazine (10 mg/kg BW) followed by a intrascapular and right midclavicular incision at the level of the carotid sheath. Blunt preparation was used to create a subcutaneous canal between the two incisions. Subsequently, the vena jugularis dextra was located and a catheter (2Fr, PV 10 cm, Instech Labs) was inserted and gently pushed forward into the vena cava superior, where it remained for the length of the study. The catheter was ligated to the vein using Ethibond Excel 4.0. The distal end of the catheter was connected to a button (25 G, VAB, Instech. Labs), which was placed subcutaneously in an intrascapular position for easy access. After verifying that there was no leakage, the incision sites were closed with Ethibond Excel 4.0. Through the catheter, the mouse was treated with heparin (15 I.U., Sigma-Aldrich) and an antibiotic mix of Ticarcillin (20 mg booster, Sigma-Aldrich) and Amikacin (10 mg/kg BW, Sigma-Aldrich). Animals received Ketoprofen (5 mg/kg BW, Sigma-Aldrich) once daily during a 3-day recovery phase. Afterwards, mice were put on a caloric restriction diet (85% w/w of regular 24-hr consumption) 3 days prior to start of the experiment. We preconditioned animals on sucrose pellets in a 60-min session once a day for 7 consecutive days, whereby animals learned to nose-poke the active portal in a self-administration chamber in order to receive food reward. The number of nose-pokes

required to get a sucrose pellet escalated starting with a fixed ratio of 1:1 (FR1) up to a fixed ratio of 5:1 (FR5). Only animals that had successfully been conditioned on sucrose pellets advanced to the testing phase, during which they administered nicotine to themselves through the implanted catheter. Catheter patency was verified daily by aspiration of blood and subsequent heparin infusion. Animals with blocked or dislocated catheters were excluded from the study. The self-administered nicotine doses started with 0.03 mg/kg/injection for 4 days, then 0.1 mg/kg/injection for 8 days, 0.25 mg/kg/injection for 8 days, and 0.4 mg/kg/injection for 8 days. The number of nose-pokes of the active versus the inactive portal, as well as the number of injections administered, were recorded and analyzed using GraphPad Prism 7.0 and multiple t-tests with Holm-Sidak correction. Survival was plotted as a Kaplan-Meier curve with significance levels calculated using modified Chi-square tests (Log-rank and Gehan-Breslow-Wilcoxon).

Cotinine assay: Blood of F1 males from nicotine-exposed or control fathers was collected in EDTA-coated tubes after injection of 1.5mg/kg nicotine free-base i.p. at 15 min, 30 min, and 45 min post-injection. Cellular components were separated from serum by centrifugation at $12,000 \times g$ for 10 min. Cotinine levels in serum of chronic F1s were measured using a Direct ELISA kit (CalBiotech Inc.). Samples were run as 2 technical replicates together with a cotinine standard curve for each 96-well plate. Analysis was performed using GraphPad Prism 7.

Anxiety assays: The elevated plus maze consisted of four arms connected by a central axis (5 x 5 cm) and was elevated 45 cm above the floor. Two of the arms contained plastic black walls (5 x 30 x 15 cm) while the other two remained open (5 x 30 x 0.25 cm). Mice were individually placed on the center of the maze with their heads facing one of the open arms and allowed 5 min of free exploration. The number of entries into the open and closed arms, and the total time spent in the open and closed arms was measured by MED-PC IV software (MED associates, Inc.). The apparatus was thoroughly cleaned between animals. For activity in the open field, mice were placed in a rectangular arena made of Plexiglas (40 x 40 x 30 cm) and mouse activity was video recorded for 10 min. Total activity, velocity, and time spent in the peripheral and central area of the open field was analyzed using video tracking software (Noldus Ethovision).

cFos staining and cell count: F1 males from TA- and nicotine-exposed fathers were treated as for transcriptome analysis and phenotype studies. Briefly, animals received nicotine in their drinking water (200 µg/ml nicotine free-base) for six consecutive days starting at seven weeks post-natum. Afterwards, mice were put on filtered tap water from 12:00 P.M. until 7:00 A.M. the next day followed by immediate tissue collection. Brains of additional eight-week old control animals are dissected 90 min after i.p. injection of 1.5 mg/kg BW nicotine free-base. Animals were anesthetized with sodium pentobarbital i.p. (200 mg/kg BW) followed by intracardial infusion of 10 ml ice-cold PBS and 10 ml paraformaldehyde (PFA; 4% w/v in PBS). Brains were kept at 4 °C in 4% PFA for 2 hr and then transferred into 30% sucrose (w/v in PBS) until slice preparation.

Brains were sectioned using a microtome (Leica) into 25- μ m slices and immersed in a 50% glycerol, 50% ethylene glycol solution (Sigma) to preserve the tissue. Brain slices were stored in -20 °C until further processing. Using the free-floating immunostaining method, slices were washed with PBS for 5 min, permeabilized with 0.5% (v/v) Triton X-100 (Sigma) for 10 min, and blocked with 3% donkey serum for 30 min. The slices were incubated overnight at 4 °C with antibodies against c-Fos (1:1000, catalog number: sc-52, lot number: D2315, Santa Cruz Biotechnology, Santa Cruz, CA). After washes with PBS, slices were incubated with Alexa Fluor 594 secondary antibodies (1:1000, ref number: A21207, lot number: 1602780, Life Technologies, Carlsbad, CA). Counterstaining was carried out with DAPI through mounting media (Cat number: H-1200, lot #: ZB0730, Vector, Burlington, CA). Fluorescent images were captured using an AxioCam MRm camera (Carl Zeiss, Peabody, MA) attached to a Zeiss Axiovert inverted fluorescent microscope equipped with Zeiss filter sets 38HE, 49, and 20. Zeiss objectives A-p were subsequently processed using Axiovision version 4.8.2. Quantification of c-Fos-positive cells was performed using ImageJ, with a minimum of 6 hippocampal brain slices analyzed per animal.

Tissue harvest for hippocampal mRNA-Seq: Seven week-old male F1 animals from control (TA) and nicotine-exposed fathers were divided into three treatment groups: naïve, chronic, and chronic + stimulation. Naïve mice were not exposed to nicotine before tissue collection at 8 weeks of age. Chronic animals received nicotine in their

drinking water (200 ug/ml) for six consecutive days. Afterwards, chronic mice were put on filtered tap water from 12:00 P.M. until 7:00 A.M. the next day followed by tissue collection as for naïve animals. Chronic + stimulation animals were treated as chronic animals, but received an additional nicotine injection (1.5 mg/kg BW nicotine free base i.p.) 30 min before organ harvest. For all three sets of animals, following sacrifice brains were explanted and put on ice. A midline incision was executed and midbrain, hypothalamus, and hippocampus of either side were dissected. Tissues were immediately immersed in liquid nitrogen, then stored at -80 °C until further processing.

Hepatocyte isolation for mRNA-Seq and for ATAC-Seq: Eight week-old male F1 animals from control (TA) and nicotine-exposed fathers were anaesthetized using ketamine (100 mg/kg BW) and xylazine (10 mg/kg BW). The abdominal cavity was opened with a transverse incision below the rib cage. The portal vein was dissected with blunt forceps and a 26 G catheter needle was inserted. After cutting the vena cava inferior cranial of the liver, the organ was perfused firstly with 1X HBBS + 200 mM EDTA (10 ml at 7 ml/min) and secondly with 50 ml DMEM containing collagenase type I (0.4 mg/ml) at 7 ml/min. The liver was then removed from the abdominal cavity, put in a petri dish containing culture medium (DMEM, 20% FBS, 1X ITS, 1X Penicillin/Streptomycin, 0.1 uM Dexamethasone, Sigma-Aldrich), and gently dissected to allow release of hepatocytes and supporting cells from connective tissue. Note that due to the disaggregation of the entire liver, mRNA abundance changes observed in a subset of hepatocytes (such as, for example, dying cells in drug-acclimated animals – **Figure 2.14**)

will be diluted out by the majority of unaffected hepatocytes. After filtration through a 70 μm nylon cell strainer, cells were washed twice with PBS 1X and once with culture media (centrifugation at 500 rpm for 5 min), and plated on a 0.1% gelatin-coated well.

Hepatocytes were allowed to adhere to the bottom of the well for three hours.

Nonadherent cells were then removed, and fresh culture medium (DMEM, 20% FBS, 1X ITS, 1X Penicillin/Streptomycin, 0.1 μM Dexamethasone, Sigma-Aldrich) was then added, initiating our time course (T0, T1, T3, T21 hours) at 37° Celsius and 5.0% CO₂.

Cells were collected after a PBS 1X wash by adding TriZol to the well for RNA experiments.

RNA-Seq: Strand-specific libraries were prepared as previously described (Zhang et al., 2012). Briefly, brain and liver were collected from nicotine-exposed and control F1 males. Hepatocytes were isolated as described above. For the hippocampus, after sectioning of brain into 1-mm slices, areas of interest were identified according to the Mouse Brain Atlas by Paxinos and Franklin and dissected using 0.5-mm punches.

RNA from brain and liver was isolated using standard TriZol protocols, followed by rRNA depletion (RiboZero kit, Illumina, Inc.). After first- and second-strand synthesis, adapters were ligated to fragments and amplified using multiplexed PCR primers.

Libraries were sequenced on a NextSeq 500 platform from Illumina, Inc. Quality-controlled reads were aligned to the reference genome (*Mus musculus/mm10*) with Bowtie2 and differential expression was calculated using DESeq2. For multiple

comparison adjustments, we used Holm-Bonferroni correction as a more conservative approach.

RNA-Seq data are available at GEO, accession # GSE94059.

ATAC-Seq: ATAC-seq libraries were prepared for 16 hepatocyte samples (4 NIC and 4 TA animals, with each sample split into untreated and dexamethasone-treated aliquots) as previously described (Buenrostro et al., 2015) using the Nextera DNA Library Preparation Kit (Illumina). Libraries were paired-end sequenced on a NextSeq 500, and reads were aligned to mm10 using Bowtie2, v2-2.1.0 with the parameters -D 15 -R 2 -N 1 -L 20 -i S,1,0.50 --maxins 2000 --no-discordant --no-mixed. Mitochondrial DNA and random chromosome mapped reads were removed, and PCR duplicates were removed. Genome browser images were generated from merged datasets with reads extended to 150 bp, and normalized by total mapped reads per sample. For differential peak analysis, HOMER was used to identify NIC-specific peaks using TA peak files as background.

ATAC-Seq data are available at GEO, accession # GSE92240.

Liver histology: Livers were harvested from F1 males from nicotine-exposed and control fathers under various conditions (pre-treatment with nicotine 1.5 mg/kg BW intraperitoneal b.i.d. for 5 days or cocaine 15 mg/kg BW intraperitoneal b.i.d. or acetaminophen 400 mg/kg BW q.d. for 1 day) and washed with PBS. A 4-mm slice was

taken from each lobe and put in ice-cold 4% formaldehyde overnight. The next day, samples were dehydrated in a series of escalating ethanol solutions starting with 70% and ending with 100%, embedded in paraffin, and sectioned (4 μ m slices), each section containing all four lobes, which were then mounted onto a glass slide. For H/E staining, slices were de-parafinized, incubated with xylene and a series of descending ethanol solutions. Incubation times for Mayer's hematoxylin (Sigma-Aldrich) and 1% Eosin Y (Sigma-Aldrich) were 30 sec and 20 sec, respectively. After dewaxing of tissue, TUNEL staining was performed following the manufacturer's recommendations (in Situ Cell Death Detection Kit, POD, Roche). Apoptotic areas per lobe were counted under a light microscope with 20X magnification at five different levels through the sample and analyzed with Image J.

CHAPTER III: DISCUSSION

Study Summary

Although the field of intergenerational inheritance of acquired traits has made major contributions to our understanding of the kind of information that entire organisms can potentially pass on to subsequent generations, it has remained elusive whether exposure to different environmental circumstances in one generation can impact offspring in a very specific manner depending on the stimulus the parental generation experienced, or more broadly in terms of a general metabolic response to conditions that are sub-optimal in the development of an organism. By investigating intergenerational paternal nicotine exposure as an example of a specific receptor-ligand interaction that has a major public health impact as one of the more common drugs of abuse, we were able to show that

1) Paternal nicotine exposure can have a far reaching intergenerational impact in so far as it affects the survival of male offspring after LD₅₀ application of drugs of abuse. In our mouse model, the effects on the next generation were restricted to male mice only. The phenotype was not associated with an alteration in addictive behavior such as self-administration of the drug of abuse or addiction-related behavioral traits such as fear and anxiety response, but appeared to give offspring of nicotine-exposed fathers a fitness advantage as an overall increased survival rate on a population level, when re-introduced to nicotine or similar compounds such as cocaine.

2) The phenotypic effects of paternal nicotine exposure on male offspring were only evident after the F1 generation had been exposed to nicotine or other drugs of abuse themselves, which argues for (epi)genetic regulation that is dependent on at least two conditions, sufficient exposure to an environmental toxin in the paternal generation and re-exposure to the same or similar compounds in the next generation. We also observed a more general, primed XPG expression state of hepatocytes in naïve NIC offspring prior to drug exposure, which we speculate could lower the transcriptional threshold to allow them to respond to re-introduced changes in the environment more quickly.

3) Our paradigm of chronic paternal nicotine exposure revealed a non-specific, metabolic response in offspring and not, as was our hypothesis, a specific addiction phenotype mediated through nAChRs either centrally or in the periphery. This means that information about environmental conditions during one generation as investigated in our experimental paradigm are passed on to subsequent generations as a general quality-of-life experience, which can then prime the offspring to be better equipped to deal with such adverse conditions upon re-exposure in the future.

4) Our model of paternal nicotine exposure offers the scientific community a better-defined paradigm than the general diet-based approaches, as it focusses on a specific receptor (nAChR) ligand (nicotine) interaction. This can be useful for a variety of experiments in the future, which require eliminating confounding factors, and can help to dis-entangle the extent, to which various factors contribute to an intergenerational phenotype.

Contextual Interpretation of Results

In the following section, we shall provide scientific background and context that may be helpful in the interpretation of our data. Although we performed an extensive screen in F1 offspring to establish a robust read-out for our paternal nicotine exposure paradigm, there are unanswered questions that remain left to be solved by future experiments. This section may also assist in the composition of future studies by considering what has been done by others in the field. After all, “*nos esse quasi nanos gigantum umeris insidentem*” as Bernard of Chartres once said.

Nicotine-specific versus non-specific F1 Phenotype

Much to our surprise, specific nicotine-mediated stimulation of nAChRs in the paternal generation did not result in a nicotine-related behavioral phenotype in the F1 generation. However, it is worth noting that we performed only a limited number of behavioral tests. We investigated the rewarding potential of nicotine in offspring coming from nicotine-exposed fathers compared to control offspring in a classic self-administration experiment, which is the gold standard in the field. We also took a closer look at potential differences in the somatic effects of nicotine in the F1 generation as assessed by locomotor activity over time. Finally, we studied potential phenotypes of nicotine that are related to anxiety-spectrum disorders with elevated plus maze experiments. In none of these behavioral tests did we find any evidence that would allow us to conclude that paternal nicotine

exposure has a specific nicotine-mediated effect on the F1 generation. However, we did not test memory or learning in F1 offspring. It is known that exposure to nicotine for a short amount of time can improve short-term memory both in animal models of schizophrenia (Hambusch et al., 2014) and human cohort studies, in which participants received either nicotine gum (Phillips and Fox, 1998) or transdermal nicotine patches (Poltavski and Petros, 2005) (Froeliger et al., 2009). These effects are probably mediated by the homomeric $\alpha 7$ subtype of nAChRs in the hippocampus (Hambusch et al., 2014; Weiss et al., 2007). While we have no phenotypic or behavioral evidence for a hippocampal connection, RNA sequencing studies of the hippocampus of offspring from nicotine-exposed and control fathers revealed no significant changes on the transcriptional level, specifically for neither $\alpha 7$ nor other nAChR subunits, but also over the entire transcriptome, and, therefore, suggest that the hippocampus and memory-related functions in F1 offspring may not be the major target of intergenerational nicotine effects.

It should be noted that other studies have found such differences in complex cognitive learning abilities in the F1 generation of nicotine-exposed parents. In particular, there is one study performed in rats (Renaud and Fountain, 2016) that is of interest. F1 generation rats that came from nicotine-exposed parents and were exposed to nicotine themselves during adolescence did worse in special recognition and learning experiments than F1 rats that received nicotine during their adolescence, but came from parents that had not

been exposed to nicotine (control). This study, however, has many flaws. Firstly, it exposed both male and female parents to nicotine prior to mating, thus may have confounded effects of nicotine that are transmitted through the paternal side of transfer versus maternal effects. Secondly, in contrast to our study, the researchers did not investigate the molecular level of intergenerational nicotine exposure, but focused their attention on only the behavior. Without having an additional layer of evidence such as changes on a cellular or molecular level, a sample size of 15 test subjects per condition is far too small to make claims about intergenerational phenotypes. To achieve a statistical power of 80% and assuming an effect size of 0.5, one would have to include at least 50-60 animals per group to be able to reliably calculate statistical significance. Thirdly, nicotine was administered to the animals through intraperitoneal injection twice a day, which could in itself act as a major stressor and elicit behavioral and molecular changes that can be passed on to the next generation. Thus, it is conceivable, that the observations made in the F1 generation have nothing to do with nicotine, but rather are the results of a series of physical injuries that resulted in a general stress response in these animals. This is one reason of why we decided to administer nicotine to the animals in their drinking water, as this reduces the impact of confounders such as external or concomitant stressors introduced, for example, by i.p. injections. It is, however, nice to see that even at this level of statistical trends, there is a “two-hit requirement”, i.e., both the parental generation and the F1 generation have to be exposed to nicotine to see an effect, which is what we observed in our paradigm, as well.

A much better designed study that drew a lot of attention in the scientific community showed a receptor-ligand specific effect in offspring as response to paternal fear conditioning using a specific odor, acetophenone, combined with electric foot shocks (Dias and Ressler, 2014). Surprisingly, both the F1 and F2 generations showed increased sensitivity to this odor, which could morphologically be traced back to a specific olfactory receptor (Olf151) in the brain with hypo-methylation of the Olf151 region in sperm of F0 and F1 males. As interesting as these findings appear to be at first glance, a major flaw of this experimental paradigm is again the small sample size. Researchers, who conducted the study, used a sample size of only 4 for their methylation analysis. Our lab has shown in the past that small sample sizes lack statistical power, as they cannot differentiate between true positives and false over-representation of a certain sub-population within the relatively wide methylation variability due to intrinsic epi-variation of the methylation state of specific genomic regions (Shea et al., 2015). On the other hand, this olfactory receptor study validated the observed receptor-ligand specificity with *in-vitro* fertilization (IVF) experiments, in which sperm from either a stimulus-exposed or control father was injected into a control oocyte and the embryo transferred into a pseudo-pregnant control foster female. Nevertheless, the findings of this study stand rather alone in a sea of support in favor of a more generalized offspring response.

Gender Difference in F1 Phenotype

Before we discuss potential reasons for the gender-associated difference in F1 phenotype expression, it shall be noted that phenotype preferences of one or the other gender are commonly seen in diseases such as cancer, genetic disorders such as mutations of metabolic pathways, or multifactorial pathologies such as cardiovascular diseases, neurological, and psychiatric disorders. It is, however, of utmost importance to understand, what the underlying causes for these gender differences might be, even more so in the context of intergenerational epigenetics, where special caution is required to account for a plethora of potential confounders. The observed phenotype in the F1 generation is only detectable in male offspring from nicotine-exposed fathers. Initially, we tested both males and females. The locomotor activity assay did not show a pronounced phenotype in female F1s from nicotine-exposed fathers. When we performed the nicotine self-administration experiment to study the rewarding effects of nicotine in the F1 generation, it became clear that female mice at an age of seven to eight weeks *post-natum* are not readily trainable in the nicotine self-administration paradigm. It is not so much the nicotine administration itself that causes the issue, but rather the fact that female mice cannot be conditioned to reproducibly prefer the active portal over the inactive portal during the training with sucrose pellets. The preference of the active portal over the inactive portal should at least lie in the range of 1:5 to 1:10 (Fowler and Kenny, 2011), which we did not achieve in female test subjects, so they could not be included in this study. In addition to those negative results, naïve females did not display a survival difference between F1s from nicotine-exposed fathers and control fathers. This let us

conclude that there is no obvious behavioral phenotype in F1 females from nicotine-exposed fathers in our experimental model and in the conditions that we tested. It is not uncommon that transgenerational studies reveal a sex preference of the observed phenotype in subsequent generations. There are many possible explanations for this phenomenon, of which only a few shall be mentioned.

Firstly, the expression of a certain phenotype could be dynamic over generations rather than a sharp yes-or-no answer. This means that it is completely conceivable that a certain phenotype could be expressed more quickly in one gender, but needs the persistent exposure of several consecutive generations in the other sex. In this scenario, sex differences are not true positives, but depend on experimental conditions such as the length of observation post-exposure, the level of granularity of what is considered a phenotype, and the dynamic resolution capacity of equipment used to observe a particular phenotype. For example, changes in the expression level of genes may serve as a phenotypic biomarker for a molecular biologist, but have less meaning to a behavioral scientist, if these transcriptional changes do not result in an alteration of behavior in the test subjects at a given time point, when the test is performed. Thus, in this case, gender differences are observation-based.

Secondly, it is possible to imagine a scenario, in which intergenerational traits are not completely penetrant on a population level, but rather need to pass a certain threshold in

an individual to display a phenotype that is readily observable. In our case, the differences in hormone levels between males and females could raise the bar for females to show the exact same liver phenotype that we see in males. We found in our ATAC-Seq data that Nuclear Hormone Receptor (NHR) responsive elements in the genome are more accessible in male offspring from nicotine-exposed fathers. We did not look at female F1s, but it is known that, for example, estrogen and estrogen receptor alpha are liver-protective in that they stimulate mitochondrial function and metabolism (Madak-Erdogan et al., 2016). Estrogen also increases the expression of NHRs such as Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ) and Nuclear Respiratory Factor 1 (NRF1) in liver and other tissues (Hamilton et al., 2016; Ribas et al., 2010). As males have very low levels of estrogen under physiological conditions, this difference and the altered regulation of associated pathways could account for the observed gender gap. Additionally, growth hormone can induce nuclear translocation of Hepatocyte Nuclear Factor-4alpha (HNF-4 α), Pregnane X Receptor (PXR), and Retinoic X Receptor alpha (RXR α) and up-regulation of CYP3A5, an important cytochrome P450 subunit involved in metabolism of many xenobiotics, an effect that is much more pronounced in female primary hepatocyte culture than in male primary hepatocyte culture (Thangavel et al., 2013). This gender difference is also true for other cytochrome P450 subunits such as CYP3A4 and appears to be the result of an increased rate of nuclear translocation of HNF-4 α , PXR, and RXR α in females upon stimulation with growth hormone or corticosteroids (Dhir et al., 2006; Thangavel et al., 2011). An interesting fact in this context is that growth hormone release from the pituitary gland follows two very distinct

patterns in males (pulsatile with peaks every 3-4 hours in rats) and females (constant), respectively (Agrawal and Shapiro, 2001; Dhir et al., 2006; Waxman and Holloway, 2009). The same general male-peak v female-constant patterns can be observed in mice (MacLeod et al., 1991) and humans (Veldhuis, 1998). Mechanistically, calcium stimulates the release of growth hormone from cells in the pituitary gland. The calcium response of these growth hormone cells is more pronounced in males than in females with this difference being partly caused by gonadal steroids as a feedback loop in human adults (Sanchez-Cardenas et al., 2010), which again are different between males and females.

Another possibility is that metabolic pathways such as the one-carbon donor S-adenosylmethionine (SAM) pathway, which provides the donor for cytosine methylation and can be altered by stress levels (Saunderson et al., 2016), or the metabolism of alpha-ketoglutarate and Fe(II), which are involved in de-methylation processes through dioxygenase enzymes (TETs) (Ito et al., 2010; Tahiliani et al., 2009), are so different between males and females due to the higher levels of, for example, estrogen in female mice, that an altered cytosine methylation state of certain gene promoters or enhancers of xenobiotic processing or metabolic genes in the liver changes the phenotypic outcome between the two genders. All these described factors could alter the threshold of when molecular changes become detectable as a phenotype. In all these cases, observed gender differences would only be secondary to an underlying confounder or primary cause and could be resolved and standardized, when these factors are taken into account in the

experimental design, which would require a much broader mechanistic approach than what we applied in our experimental paradigm.

Thirdly, the gender differences that we saw in our study could be true. If this is the case, then the mediating entity of the observed phenotype must either lie on the sex chromosomes or as a second possibility could still be encoded on the autosomes, but would functionally regulate something that is encoded on the sex chromosomes. The former scenario is rather unlikely, as neither any of the nAChRs nor any of the CYP P450 enzymes are encoded on sex chromosomes. In fact, human $\alpha 4$ and $\beta 2$, two of the more common subunits involved in nicotine reward and addiction, are encoded on chromosomes 20 and 1, respectively, whereas in mouse they are located on chromosomes 2 and 3, respectively. The $\alpha 7$ subunit, which is expressed in the hippocampus and surprisingly also in mature sperm, is encoded on chromosome 15 in humans and on chromosome 7 in *Mus musculus*. Thus, we are left with the latter scenario, for which there is some evidence in the literature. For example, in order to compensate for the additional X chromosome in females, one of them is usually inactivated during early embryonic development in a non-random manner (Heard et al., 1997; Lyon, 1961; Tan et al., 1993), a process largely influenced by the so called X chromosome controlling element (Xce), an approximately 1.9 MB region on the X chromosome that acts in cis and amongst others comprises the long non-coding RNA Xist (Calaway et al., 2013; Chadwick et al., 2006; Thorvaldsen et al., 2012). Researchers have found that this region acts as a sponge that can bind factors needed for dosage compensation (Blewitt et al.,

2005; Calaway et al., 2013). It is conceivable that such factors, be it proteins in the form of transcription factor activators or repressors and similar, or be it small and long RNA entities, would then be under-represented in other regions of the genome, which could in turn change the expression levels of coding and non-coding sites in those regions. To provide additional support for this hypothesis, factors located on autosomes are known to be involved in the X-inactivation process, as for example *Xiaf1* and *Xiaf2* on mouse chromosome 15 (Percec et al., 2002). Again, the expression of *Xiaf1* and *Xiaf2* and their dys-regulation in the male genome, respectively, could be involved in other regulatory processes that have yet to be discovered and thus, could account for some of the gender differences that we observed in our mouse model of chronic paternal nicotine exposure.

Interestingly, the neo-natal brain is particularly prone to X-inactivation that under-represents the paternal X chromosome (Wang et al., 2010). The brain in general is an organ that presents with strong allele-specificity in the scope of imprinting. For example, the *Igf-2* locus is highly involved in adult neurogenesis in the hippocampus, particularly in the sub-ventricular zone and the sub-granular zone (Bracko et al., 2012). *Igf-2* is solely expressed from the paternal allele in neural stem cells, while the maternal allele is completely shut off (Ferron et al., 2015). It has been shown that maternal caloric restriction led to an increase in *Igf-2* expression in the hippocampus of female F1s and female F2s, but not in males (Harper et al., 2014). Again, our RNA sequencing results from male hippocampus did not show significant differences between male offspring from nicotine-exposed and control fathers, but at the same time we did not look at F1 females from nicotine-exposed and control fathers. Again, it is theoretically conceivable

that gender differences observed by us and others are based on true biological processes due to altered dosage compensation of the X chromosome or imprinted alleles in specific organs or tissues between males and females. In this scenario as well, a broader mechanistic approach is required to be able to unveil these pathways.

Survival Phenotype and Nuclear Hormone Receptor Pathways

In our experimental paradigm, we saw an up-regulation of xenobiotic processing genes (XPG) on a transcriptional level in the liver of naïve male F1 offspring from nicotine-exposed fathers compared to offspring from control fathers as determined by RNA-sequencing experiments. In a time course experiment, in which we treated PMHs with nicotine over a period of 24h, this up-regulation in NIH hepatocytes relative to control hepatocytes could be observed throughout each time point, but did not increase with the time being exposed to nicotine. It is interesting that naïve hepatocytes show a de-repression of XPGs without any previous exposure to nicotine. We made the same observation, when we looked at liver samples from NIC and control offspring. Although the differences were not as pronounced as in the *in-vitro* setting due to greater inter-individual variability, NIC offspring did display a higher baseline expression level of XPGs even in a naïve state without major differences in additional up-regulation after nicotine exposure between F1s from nicotine-exposed and control fathers. Confirmation of Cyp2a5 expression levels, the major metabolic subunit for nicotine, in liver samples from NIC (n=6) and control (n=4) offspring by qRT-PCR showed significant up-

regulation of this gene in NIC offspring (**Figure 2.10C**). These results, however, raise the possibility that there are several mechanistic pathways that need to act in a sequential or synergistic manner after xenobiotic exposure to lead to the observed survival phenotype.

ATAC-sequencing data showed that chromatin was generally more accessible across the entire genome in hepatocytes from F1 males of nicotine-exposed fathers (NIC hepatocytes). In addition, we observed 1861 peaks of chromatin accessibility that were significantly higher in NIC hepatocytes than in control hepatocytes, amongst them being peaks near genes such as Nr1h3 (CAR, Constitutive Androstane Receptor) and Nr1i2 (PXR, Pregnane X Receptor). Nr1h3 and Nr1i2 belong to the nuclear hormone receptor (NHR) family and regulate expression levels of metabolic and XPG genes. CAR and PXR are especially closely related to each other. These NHRs form heterodimers with retinoid-X receptor (RXR) and thus, activate gene transcription upon translation into the nucleus from the cytoplasm. NHRs that induce the expression of Phase I and Phase II biotransformation genes such as members of the Cytochrome P450 super-family responsible for redox and hydrolytic reactions (Chen et al., 2003; Itoh et al., 2006; Kojima et al., 2007; Sueyoshi and Negishi, 2001), and glucuronosyltransferases and sulfotransferases that are involved in conjugation reactions (Sugatani et al., 2001; Xie et al., 2003), as well as multi drug transporters (belonging to Phase III of the detoxification reaction) (Burk et al., 2005; Geick et al., 2001), have a highly conserved N-terminal DNA binding domain comprising eight cysteines (Fang et al., 2012; Giguere et al., 1988) and interacting with hormone response elements (HRE) in the genome, as well as a

ligand domain with three layers of alpha-helices that are able to form such flexible and big enough a pocket to allow binding of a variety of xenobiotic molecules (Evans, 1988; Noble et al., 2006; Watkins et al., 2001). This promiscuity towards xenobiotics enables NHRs to respond to a broad spectrum of substances that get introduced into the body. For example, the well-known anti-epileptic drug phenobarbital (PB) leads to dephosphorylation of threonine 38 upon binding to CAR and its subsequent translocation from the cytoplasm into the nucleus, where it induces up-regulation of CYP2B10 expression (Mutoh et al., 2009). Induction of the Cytochrome P450 enzyme complex by PB can affect the bio-availability of other drugs and compounds as well, as they use the same bio-transformation pathways and thus, will be metabolized and removed from the system more quickly.

In our experimental paradigm of primary hepatocyte culture, however, CAR and PXR are up-regulated at a naïve state in NIC hepatocytes compared to control and no further up-regulation is observed upon *in-vivo* nicotine or cocaine stimulation prior to the perfusion and hepatocyte isolation. Interestingly, the observed difference on a transcriptional level *in-vitro* does not translate into an *in-vivo* phenotype, as naïve animals do not show a survival difference, when challenged with a variety of nicotine concentrations ranging from very low to very high (dose-response curve). An alteration in the survival rate of F1 males from nicotine-exposed fathers is only detectable in animals that were chronically exposed to low levels of nicotine prior to the LD₅₀ challenge (chronic F1 cohort) and, at the same time, whose hepatocytes do not increase gene expression of cytochrome P450 or

NHR genes much further beyond what is seen in naïve NIC hepatocytes. This discrepancy between what is seen on a transcriptional level and what ultimately amounts to a measurable phenotype could not be resolved in our experimental paradigm. *In-vivo* analysis of liver samples showed large variability, which is not surprising at all considering that these intergenerational effects probably only manifest on a population level as a whole, i.e. the penetrance is most certainly not 100%. To phrase this argument differently, if intergenerational effects were so penetrant that they could affect every single individual of a population coming from a father that had been exposed to a particular stimulus, then every minor single change in the environment could potentially be so detrimental and lethal to an entire species, that we as human beings would not be around anymore to perform this type of experiment. Inter- or transgenerational effects can – *per definitionem* – only be minor and be of significance only for a population in its entirety. Thus, we speculate that there was probably an evolution of cellular and organismal response mechanisms that are able to suppress whatever effect the environment may have, similar to how piRNAs are able to silence transposable elements, which were introduced into the genome as parasitic entities, to ensure integrity and stability of the germline and, by doing this, propagate the fitness of the gene pool. This may be the reason why effects of paternal nicotine exposure were more pronounced in *in-vitro* hepatocyte cultures than in *in-vivo* livers. In tissue culture, there are no supra-cellular control mechanisms, but only the cells themselves that have to respond to whatever stimulus is imposed directly upon them. It is also worth noting that culture medium for hepatocyte primary culture contains dexamethasone to improve survival of

cultured cells, which in itself has been shown to activate Cytochrome P450 enzymes and inhibit hepatocyte apoptosis (Hunter et al., 2017; Zhao et al., 2015). The dexamethasone effect could, thus, mask the additional benefit of low level chronic exposure to nicotine or cocaine in those hepatocytes. To further investigate the influence of dexamethasone on primary hepatocyte gene expression levels and survival, control experiments without dexamethasone can be performed simultaneously to the usage of standard culture conditions. As a summary, it is not unexpected that *in-vitro* primary hepatocyte conditions do not exactly replicate what happens *in-vivo*.

Our TUNEL assay that stains apoptotic cells and the associated finding that F1 males from nicotine-exposed fathers show significantly lower levels of apoptotic cells around the central vein than control F1 males, but only after chronic exposure to cocaine, but not nicotine, indicate that there must be additional factors that contribute to the F1 survival phenotype that could, when discovered, explain the discrepancy between transcriptional level and phenotype. It is a known fact that cocaine is hepatotoxic (Thompson et al., 1979), mostly mediated through its main metabolites norcocaine, norcocaine nitroxide, and N-hydroxynorcocaine (Ndikum-Moffor et al., 1998). It also is not surprising that our TUNEL assay post chronic nicotine exposure did not result in apoptotic cells in the liver after nicotine exposure, as nicotine is not known to be hepatotoxic. We, therefore, focused on cocaine-mediated hepatotoxicity to determine differences in cell survival between groups. It is, however, remarkable that chronic cocaine exposure in F1 males from nicotine-exposed fathers resulted in significantly reduced hepatotoxicity of cocaine

and a higher survival rate of NIC offspring after short-term exposure to either nicotine or cocaine followed by LD₅₀ of nicotine or cocaine. Extrapolating the survival phenotype on an organismal level, we speculate that altered gene regulation could result in a survival phenotype on a cellular level, i.e., the more hepatocytes survive low level exposure to a xenobiotic, the more cells can metabolize the very same xenobiotic, when it is applied as an LD₅₀ dose. This hypothesis is supported by the fact that male offspring from nicotine-exposed fathers metabolize nicotine into cotinine faster than control offspring. It will be important to expand the histologic assessment of liver samples to the quantification of necrotic areas in the liver, as they may show a different distribution than what we saw in our TUNEL assay. Acetaminophen is highly hepatotoxic and causes necrosis in the liver after a single dose of 400 mg/kg BW. As Acetaminophen is a non-steroidal anti-inflammatory drug (NSAD), determining the phenotypic response in livers from NIC and control F1s will help to address the underlying mechanisms beyond the drugs of abuse that were used in this study.

As CAR and PXR also metabolize endobiotics, it is scientifically conceivable that other endogenous hormones, such as estrogen as the major sex hormone in females, could have an additional impact on the response of NHRs to xenobiotics such as nicotine, alter survival outcome post nicotine LD₅₀ applications, and again provide an explanation for the gender differences that we described above. Furthermore, the sympathetic nervous system plays a prominent role in hypothalamic stimulation and pituitary release of growth hormone and thus, influences the level of corticosterone and testosterone in the blood

circulation (Kot and Daniel, 2011; Sadakierska-Chudy et al., 2013), which in turn have an impact on cytochrome P450 expression, as was elaborated above. In addition, corticosteroids such as dexamethasone increase hepatocyte survival in culture through the same CAR, PXR, RXR pathways. Following the argument that the hypothalamus-pituitary-adrenal axis (HPA axis) impacts cytochrome P450 expression through growth hormone, corticosteroids, and NHR interactions, the increased survival in F1 males from nicotine-exposed fathers after short-term F1 exposure to a certain drug of abuse could have a general stress response as the underlying mechanism. Further experiments need to be performed to determine hormone levels in F1s before and after exposure to nicotine or cocaine. But it is a viable hypothesis to assume that the non-specific survival phenotype that we detected in our experimental paradigm is the result of an altered regulation of the NHR and the HPA axis.

Potential Mechanisms of F1 Phenotype

Our phenotypic study of behavioral and molecular changes in F1 males from nicotine-exposed fathers compared to control offspring revealed that

1) naïve NIC offspring show up-regulation of XPGs in the liver and in PMH culture *in-vitro* and

2) NIC offspring survive LD₅₀ doses of nicotine or cocaine at a higher percentage only after pre-exposure to either one of these drugs of abuse.

The discrepancy between the transcriptional results and the observed functional phenotype could not be resolved in our study. Nevertheless, we speculate that there may be several underlying mechanisms that could account for the missing link between transcriptome and behavioral phenotype.

Firstly, Cotinine-ELISA data reported in this work strongly suggest that NIC offspring are, indeed, able to functionally metabolize nicotine more quickly and, thus, clear the system of harmful toxins more effectively than control offspring, despite the lack of additional transcriptional changes in nicotine or cocaine pre-exposed animals. As stated previously, Cyp2A5 is the enzymatic subunit in mice that metabolizes nicotine (Li et al., 2013). We hypothesize that the raised expression level of XPGs in a naïve state could be sufficient to lead to higher levels of functional protein, which in turn could more readily metabolize nicotine, when it is introduced into the organism. As there is not always a

good correlation between mRNA abundance and translated protein levels due to post-transcriptional and translational regulatory mechanisms (Fagerberg et al., 2014; Ingolia, 2014), it will be important to employ techniques such as ribosomal footprinting to determine translation rates and further substantiate the claim that an increased baseline expression of XPGs can result in more active protein without additional up-regulation of gene expression in the pre-exposed state. In addition, Cytochrome P450 activity assays will allow us to investigate the level of functional protein in NIC and control PMHs. Depending on the half-life of these cytochrome P450 proteins, it is reasonable to think that they could remain in the cell for quite some time after translation, which would decrease the need to continuously transcribe XPGs after having accumulated a sufficient baseline amount of active protein.

Circular RNAs (CircRNAs) could serve as another way for cells to stabilize transcription products by circularizing their 5' and 3' ends and store them in the cytoplasm until they are used for translation (Granados-Riveron and Aquino-Jarquin, 2016; Memczak et al., 2013). Using the above mentioned ribosome footprinting technology, researchers have recently shown that circRNAs are indeed associated with active ribosomes, thus raising the possibility that these circRNAs are transcribed into functional proteins (Pamudurti et al., 2017). XPG mRNA products may be stored as circRNAs in a cell until additional stimuli such as the exposure to nicotine or cocaine activate translational pathways. Furthermore, circRNAs have been shown to display regulatory functions. Sex-determining region Y (SRY), for example, can be present as circRNA in testes that can act as a sponge for microRNA-138 (miRNA), thus affecting regulatory signals that these

miRNAs may otherwise promote (Hansen et al., 2013). It is possible that circRNA-based mechanisms regulate XPGs post-transcriptionally. CircBase (www.circbase.org) is an online database developed by Nikolaus Rajewsky and his lab in collaboration with the Max-Delbrück Center for Molecular Medicine in Berlin, Germany, that provides an excellent catalogue of all known circRNAs and also serves as a prediction tool for new circRNA entities (Glazar et al., 2014).

As small RNAs such as miRNAs have been shown to regulate Cytochrome P450 subunit expressions (Zanger and Schwab, 2013), an additional investigation of small RNA profiles in NIC versus control PMHs may contribute to a better mechanistic understanding of the observed phenotype. PXR, for example, has a miRNA recognition site in its sequence, to which miRNA-148a can bind and inhibit PXR translation, thus changing the level of active CYP3A4 in human liver (Matsuda et al., 2008). As our nicotine time course experiments in PMH culture did not lead to additional changes in Cytochrome P450 or NHR mRNA levels beyond the ones that are already present at baseline, circRNAs and small RNAs could provide further insight into the downstream regulatory mechanisms that ultimately lead to increased survival in NIC offspring after a challenge with high doses of nicotine or cocaine.

Secondly, in addition to a significant up-regulation of XPGs in naïve NIC PMHs, we also observed changes in metabolic genes, most prominently within lipid metabolism pathways (**Figure 2.9E**). These findings further substantiate the assumption that F1

offspring may respond to paternal nicotine exposure in a rather non-specific manner. However, comparing the lipid metabolism genes that are affected in our nicotine paradigm with the ones that displayed major changes in a previous study from the Rando lab, in which the paternal generation was exposed to low-protein or control diet (Carone et al., 2010), we saw little to no overlap. The diet paradigm resulted in lipid metabolism gene changes that mainly affected the cholesterol synthesis pathway, whereas in our study metabolic genes, whose expression levels were significantly altered, are involved in a variety of lipid metabolism pathways, but not in cholesterol synthesis. Although this comparison appears to be intriguing at first, it shall be noted that the experimental design of the two paternal exposure paradigms was quite different. In Carone et al., microarray analysis was performed on dissected livers from 3-week old F1 pups that had not been weaned from mother's milk yet, while our nicotine study performed RNA sequencing experiments on 8-week old, mature F1 animals that had been on regular diet for five weeks. As pre- and post-weaning liver metabolism differs rather significantly (Renaud et al., 2014), no meaningful conclusions between these two datasets can be drawn. We, instead, investigated the response of F1 males from nicotine-exposed and control fathers to glucose and insulin injections and observed a trend towards insulin-hyposensitivity in NIC offspring (**Figure 2.10A,B**). As the sample size in this experiment was small, we advise caution in interpreting these data. On the other hand, it has recently been shown that NHR pathways can, indeed, regulate the storage of fat-droplets in *C. elegans* (Li et al., 2017). Peroxisome Proliferator-Activated Receptors (PPARs) are a sub-family of NHR with known functions in metabolic homeostasis, lipid, and glucose metabolism

(Montagner et al., 2016; Regnier et al., 2017). PPARs often function as heterodimers with other NHRs such as Retinoic acid receptor-related Orphan Receptor alpha (ROR α) (Kim et al., 2017) or RXR to ensure metabolic homeostasis (Evans and Mangelsdorf, 2014). As described above, PXR and CAR also need to form heterodimers with NHRs such as RXR to translocate into the nucleus and initiate gene expression upon DNA binding. In our ATACseq experiments, RXR α showed increased signal in NIC hepatocytes, so RXRs can be imagined as the promiscuous binding partner to a variety of NHRs. As NHRs function in a complex network of interactions with each other, we hypothesize that the non-specific phenotype response in F1 males from NIC fathers is not limited to the mere processing of nicotine or cocaine, but also includes basic functions of metabolic pathways such as the observed up-regulation of lipid metabolism genes. Future studies will focus on this aspect of our transcriptional analysis and try to understand the network biology behind it.

Thirdly, we speculate that a primed state of increased XPG and NHR expression will lead to increased survival of hepatocytes, when exposed to drugs of abuse. Interestingly, Liver X Receptor (LXR) showed increased ATAC signal in NIC hepatocytes. PPAR γ is the heterodimeric co-receptor for LXR (Oberkofler et al., 2003). As such, LXR displays anti-inflammatory activity (Spillmann et al., 2014; Zelcer and Tontonoz, 2006), which can mitigate pro-inflammatory challenges (Ghisletti et al., 2007). It is possible that the up-regulation of LXR and PPAR γ protect hepatocytes from NIC offspring against

inflammation and subsequent cell death. The decrease in apoptosis after cocaine exposure in livers from NIC offspring as determined by TUNEL staining could indicate that this protective effect may apply to hepatocytes in a much broader manner including various drugs of abuse or other xenobiotics. A challenge with acetaminophen did not result in significant differences in apoptosis between the two cohorts, but considering the non-specific nature of our F1 phenotype there may as well be a difference in the overall level of necrosis that we did not assess in our study. If, for example, NIC offspring showed lower plasma levels of pro-inflammatory markers such as CRP, γ GT, and aminotransferases (ALT, AST) after a xenobiotic challenge, this could indicate a protective effect of NHRs that is not necessarily detectable on the histologic level of cell death, as these hepatocytes may never progress to this very late stage of inflammation-mediated response. In this study, we did not look at intermediate states of inflammation that could ultimately lead to cell death. It will be of utmost importance to perform *in-vitro* cell survival assays and measure pro- and anti-inflammatory cytokines in culture medium supernatant and *in-vivo* in the presence or absence of NHR agonists to gain more insight into the link between NHR and XPG up-regulation and improved cell survival as observed in our IHC approach.

Limitations of this Work

Despite the novelty of this study's findings, there are a few shortcomings that may have prevented us from fully uncovering the underlying mechanisms of the increased survival phenotype in F1 males from nicotine-exposed fathers after short-term F1 exposure to a drug of abuse.

1) In order to address the observed gender difference, we should have included female test subjects in our primary hepatocyte *in-vitro* assay. This could have allowed us to compare transcriptional profiles between males and females and link them to the observed phenotype. It shall be noted at this point, that females did indeed display a metabolic phenotype in term of increased body weight of females from nicotine-exposed fathers after the pre-conditioning period with sucrose in the nicotine self-administration paradigm. The effect was not significant, but considering that we only included a small number of females due to conditioning issues of the female test subjects and the fact that, during the self-administration experiment, the 24h food intake is restricted to 85-90% of the animals' normal food intake assuming an average 25g standard mouse, this result could be worth pursuing.

2) As it became increasingly clear over the course of the study that the survival phenotype that we observed in F1 males from nicotine-exposed fathers may be associated with differential regulation of CYP P450 genes and NHR response, we should have measured hormone levels in the bloodstream both in F1 males and females. Cortisol, testosterone, estrogen, growth hormone, and others may as well be involved in establishing this phenotype.

3) Our study focused purely on the F1 generation. This is reasonable, when one takes into account the sample sizes that are required to draw robust conclusion from such difficult an experimental paradigm. It is far better to try to obtain a complete dataset from one aspect of a biological process than getting fragmentary information from several different angles of a pathway. In order to provide a mechanistic explanation for the observed phenotype not only in the F1 generation, but also on the intergenerational part of the question, it is essential to investigate potential ways of transmission of this type of information about environmental exposures from one generation to the next. As stated in the Introduction, this was not within the scope of this study, but needs to be addressed, nevertheless, in the long term.

Future Outlook

This study was able to answer some of the questions surrounding the intergenerational inheritance of acquired traits through the paternal side. We have found a generalized, non-specific, metabolic response in F1 males. Future studies will focus on the underlying mechanisms that transmit information about paternal nicotine exposure onto subsequent generations. These experiments will include *in-vitro* fertilization (IVF) and embryo microinjections to identify the entities that are responsible for information transfer independent of confounding factors within the variability of *in-vivo* conceptions.

Experiments will also include offspring of both genders and investigate more closely the influence of hormone levels on and the role of NHRs in the establishment of an F1 survival phenotype by measuring Cyp P450 enzyme activity, inflammation markers in blood plasma and *in-vitro*, and using NHR agonist and antagonists to investigate a mechanistic link between transcriptome and phenotype.

It appears to be the case, that the HPA axis is involved in this concerted response. It will be important to include neonatal steroid manipulation experiments in the scope of future research, as they can shed more light on role of the HPA axis in this phenotype. Another possibility is to use gene editing technologies such as CRISPR/Cas9 to create autosomal sites of SRY and defined sequences within Xce to further investigate the “sink” hypothesis mentioned above.

The subtle, but distinct difference in sucrose pellet self-administration in male NIC offspring without differences in total body weight between the two cohorts could indicate a central role for appetite regulation or lipid metabolism. In addition, female F1s from nicotine exposed-fathers showed a greater increase in body weight, when subjected to the sucrose conditioning phase of the self-administration paradigm. Assessing 24h food intake over a longer period of time, as well as body composition and fat percentage of total body weight could help to determine the underlying mechanisms for this observation. Future studies should also include the measurement of neonatal weight in both male and female F1 in addition to weeks 3, 4, and 5 p.n., which was shown in the Results section, and correlate this with placental weight of control P0 females that were mated with either nicotine-exposed or control P0 males.

Our study, overall, has laid the groundwork for a well-defined experimental design and a clean read-out in order to investigate intergenerational effects and phenotypes.

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