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REVIEW

Guidelines on nicotine dose selection for in vivo research

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Abstract
Rationale This review provides insight for the judicious selection of nicotine dose ranges and routes of administration for in vivo studies. The literature is replete with reports in which a dosaging regimen chosen for a specific nicotine-mediated response was suboptimal for the species used. In many cases, such discrepancies could be attributed to the complex variables comprising species-

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specific in vivo responses to acute or chronic nicotine exposure.

Objectives This review capitalizes on the authors' collective decades of in vivo nicotine experimentation to clarify the issues and to identify the variables to be considered in choosing a dosaging regimen. Nicotine dose ranges tolerated by humans and their animal models provide guidelines for experiments intended to extrapolate to human tobacco exposure through cigarette smoking or nicotine replacement therapies. Just as important are the nicotine dosaging regimens used to provide a mechanistic framework for acquisition of drug-taking behavior, dependence, tolerance, or withdrawal in animal models.

Results Seven species are addressed: humans, nonhuman primates, rats, mice, *Drosophila*, *Caenorhabditis elegans*, and zebrafish. After an overview on nicotine metabolism, each section focuses on an individual species, addressing issues related to genetic background, age, acute vs chronic exposure, route of administration, and behavioral responses.

Conclusions The selected examples of successful dosaging ranges are provided, while emphasizing the necessity of empirically determined dose–response relationships based on the precise parameters and conditions inherent to a specific hypothesis. This review provides a new, experimentally based compilation of species-specific dose selection for studies on the in vivo effects of nicotine.

Keywords Human · Nonhuman primate · Rat · Mouse · *Drosophila* · *C. elegans* · Zebrafish

Introduction

This review provides in vivo nicotine dosage information as a guideline for testing hypotheses by individual investigators on the effects of nicotine as relevant to human use of tobacco or nicotine-derived medications. Using primary references as often as possible, we provide experimental evidence for species-specific nicotine dosage ranges. The intent is not to provide an extensive review of the literature but rather to provide expert insight into and advice on in vivo nicotine dosage selection based on the cumulative years of experience of the authors. We emphasize, however, that each investigator must determine empirically the precise parameters and conditions necessary to address the hypothesis under analysis in his/her own laboratory. In addition, although the authors have tried to cover comparable issues in each section, given the current state of nicotine research, this was not universally possible for all species. Finally, the authors agreed on the objective coverage of issues currently under debate.

The review is divided into a series of sections, with the next one ("Nicotine pharmakokinetics,...") addressing issues of nicotine metabolism, pharmacokinetics and pharmacodynamics, including species-specific cytochrome P450 isozymes and nicotinic cholinergic receptors (nAChRs) on neurons. Each of the other sections focuses on an individual species and addresses nicotine dosage issues related to genetic background, gender, age, acute vs chronic exposure, route of administration, behavioral responses, and disease states, where applicable. As the underlying purpose of biomedical research is to describe the human condition, the first species addressed is humans ("In vivo nicotine dose selection in humans"), followed by a section on nicotine dosages used in nonhuman primates ("In vivo nicotine dose selection in nonhuman primates"). The subsequent sections focus on in vivo nicotine parameters specific to rats ("In vivo nicotine dose selection in the rat") and mice ("In vivo nicotine dose selection in mice"), the two most commonly used experimental species, and,

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finally, *Drosophila* ["In vivo nicotine dose selection in *Drosophila melanogaster*"], *Caenorhabditis elegans* ("In vivo nicotine dose selection in *C. elegans*"), and zebrafish ("In vivo nicotine dose selection in zebrafish (*Danio rerio*)").

Many studies on the effects of in vivo nicotine on central nervous sytem (CNS) responses and plasticity are directed at understanding the neural mechanisms mediating the behavioral responses to the drug, especially those relevant to its role in human tobacco smoking. This objective governs, to a significant extent, the correlation of doses and routes of nicotine administration utilized in neurochemical and neuroanatomical investigations with those employed in behavioral studies. It also emphasizes the correlation of blood nicotine levels in animal models with those achieved in humans, either via smoking or by nicotine replacement therapies (NRTs). If the goal of the investigation is unrelated to these modes of human nicotine self-administration and is, rather, directed toward therapeutic potential or in utero exposure, the dose ranges tolerated by humans and their animal models still provide guidelines for the selection of dose and route of administration. Prolonged plasma nicotine levels in animals that are orders of magnitude greater than those achieved in humans are indicative of either a poor experimental design, a poor animal model, or both.

Just as important, however, are the many species-specific variables that can significantly affect the dose range tolerated by animal models, such as metabolism, pharmacokinetics, physiological status, and/or psychological context. In addition, in vivo animal studies minimize experimental variables, whereas variables affecting human smoking behavior are relatively uncontrolled. As such, it is reasonable to empirically determine whichever doses elicit the response of interest. This review addresses many of the mechanisms underlying these variables, so that experimental designs can incorporate procedures to insure species-specific physiological levels relevant to the response under investigation. For example, in some published papers, the doses chosen for use in one species appear to be based on other reports using a different species (e.g., mouse milligram per kilogram doses in rat studies), resulting in excessively high plasma and brain levels in the rat that may not be physiologically relevant, unless nicotine-induced seizure or hypoxia are the intent of the study. In contrast, because of the rapid nicotine metabolism in a mouse compared to humans (see "Nicotine pharmakokinetics,...", "In vivo nicotine dose selection in humans", and "In vivo nicotine dose selection in mice"), a human-based dosaging schedule might not elicit any response to nicotine in a study using mice. As such, the measurements of a murine response to acute nicotine exposure should take into consideration the mouse half-life ($t_{1/2}$), rather than the human $t_{1/2}$, even after using a route of administration that approximates human exposure [e.g., subcutaneous (s.c.) administration for the transdermal nicotine patch and intravenous (i.v.) admistration for smoking). For each species, a dose–response relationship for a particular function should be basically determined whenever possible.

Drug form

Nicotine can be purchased as either a free base (liquid at room temperature, MW 162) or in several forms of tartrate salt (nicotine hydrogen tartrate and nicotine bitartrate) with an anhydrous MW of 462. However, the tartrate salt crystallizes as the dihydrate and, therefore, a MW of 498 should be used for calculating concentrations (see below). Although the free base is the active form, a number of publications have reported the dose based only on the salt form used. Investigators need to be aware of this inconsistency when selecting a dose or dose range from the literature. In this review, we have reported the concentrations of the salt forms as identified in the original publications, whenever possible, but have appended a calculated free base concentration in brackets when necessary. If the concentration provided herein is not followed by a bracketed dose, then free base nicotine was used in the original citation. To convert the dose reported as bitartrate salt to free base nicotine, multiply the bitartrate by (162.2/498) or 0.325. Investigators are strongly advised that nicotine doses should be selected and reported as the free base concentration for all studies.

Some useful numbers and calculations

Cigarette tobacco (containing approximately 1–2% nicotine)=1–2 g per 100 g or 0.8–1.9 mg nicotine per tobacco rod. Average human body weight (BWt): 150 lb at 2.2 lb kg^{-1}=68 kg. Therefore, an average cigarette delivers roughly 10–30 μg kg^{-1}, typically resulting in 10–50 ng ml^{-1} peak plasma levels. These concentrations can be converted to molarity by dividing by MW; e.g., blood level in nanogram per milliliter divided by nicotine in nanograms per nanomole = nanomole/milliliter or (50 ng ml^{-1})/(162 ng $nmole^{-1}$)=0.309 nmol ml^{-1}=0.31 μM. Nicotine levels in the breast milk of a smoker (or a chronically exposed animal model) are also approximately two to 3 times that in plasma (almost 100 ng ml^{-1}), primarily due to the partitioning of nicotine into the high-lipid-containing, more acidic milk.

Nicotine (free base)=162 g $mole^{-1}$ MW, whereas nicotine hydrogen tartrate=498 MW. In calculating the amount (mg) of the salt form needed to administer the

desired free base nicotine dose, the following equation is useful:

$$[dose,\ mg/kg \times BWt,\ kg \times (nicotine\ salt\ form\ MW/nicotine\ free\ base\ MW)]/injection\ volume,\ ml$$

As such, in an experimental design administering 0.5 mg kg^{-1} nicotine (free base) intraperitoneally (i.p.) in a 0.3-ml injection volume to a 300-g rat, the calculation would be:

$$[0.5\ mg/kg \times 0.3\ kg \times (498/162)]/0.3\ ml = 1.54mg/ml\ of\ nicotine\ hydrogen\ tartrate\ needed$$

Nicotine pharmacokinetics, pharmacodynamics, and receptors

Components of tobacco

The pharmacologically active form of nicotine in tobacco is predominantly (*S*)-nicotine; (*R*)-nicotine, resulting primarily from racemization during combustion, comprises only about 10% of the nicotine in tobacco smoke and is much less active pharmacologically than (*S*)-nicotine. Tobacco also contains low levels of minor tobacco alkaloids, including nornicotine, anatabine, and anabasine. These minor alkaloids have pharmacological activity, albeit generally less potent than nicotine, and may contribute to some of the pharmacologic effects of cigarette smoking. Measurement of the minor alkaloids anabasine and anatabine is one way to differentiate tobacco use from pure nicotine exposure, such as that received from NRTs (Jacob et al. 1999).

Uptake and distribution

The typical smoker absorbs systemically about 20 or 0.3 mg kg^{-1} daily, based on the average United States cigarette consumption of 17 cigarettes per day (Benowitz and Jacob 1984). Blood or plasma nicotine concentrations sampled in the afternoon in smokers generally range from 10 to 50 ng ml^{-1} (0.06–0.31 μM), with trough concentrations typically at 5–37 ng ml^{-1} (0.03–0.23 μM). After cigarette smoke inhalation, nicotine is absorbed rapidly into the lungs, resulting in a relatively high nicotine concentration in the volume of blood leaving the heart. In human studies, peak arterial nicotine concentrations can be as high as 100 ng ml^{-1} (0.6 μM) depending on how the cigarette is smoked (see "Nicotine in cigarette smoke..."), while peak venous blood levels are more typically 10–50 ng ml^{-1} (0.06–0.31 μM) (Henningfield et al. 1993; Hukkanen et al. 2005; Schneider et al. 2001). This high-arterial-peak nicotine concentration reaches the brain within 8–10 s, resulting in more intensive psychoactive effects than achieved with other modes of human consumption. The rapidity of onset of effect after taking a puff strengthens the reinforcing quality of the cigarette. Such a rapid perception of nicotine's effect also allows the smoker to titrate nicotine dose and effect on a puff-by-puff basis to optimize the drug experience (see "Nicotine in cigarette smoke...").

The mean nicotine boost after smoking a cigarette in smokers without smoking abstinence on the study day is approximately 10.9 ng ml^{-1} (0.067 μM) (Patterson et al. 2003). Blood nicotine levels peak at the end of smoking a cigarette and decline rapidly over the next 20 min due to rapid tissue distribution to all body tissues, with a volume of distribution in humans averaging 2.6 times body weight. (Benowitz and Jacob 1984; Rose et al. 1999). In the bloodstream, at pH 7.4, nicotine is 69% ionized and 31% non-ionized, with <5% binding to plasma proteins. The highest tissue affinity for nicotine is in liver, kidney, spleen, and lungs, and the lowest affinity in adipose tissue. Nicotine readily crosses the blood–brain barrier and binds with high efficiency to brain tissue as well. The levels of nicotine in skeletal muscle are similar to those in blood (Benowitz et al. 1990). Nicotine also easily crosses the placenta, entering fetal blood and amniotic fluid, and is distributed in breast milk with a milk-to-plasma ratio of 2.9 (Luck and Nau 1984).

Metabolism

The metabolism and disposition of nicotine have been reviewed recently in detail by Hukkanen et al. (2005) and are only briefly addressed here. Nicotine is metabolized by the liver to six primary metabolites and several minor metabolites in humans (Fig. 1).

The quantitatively most important metabolite in mammalian species is cotinine, and in humans approximately 70 to 80% of nicotine is converted to cotinine. Other major metabolites include nicotine-*N*′-oxide, nicotine glucuronide, and the subsequent metabolites of cotinine: cotinine glucuronide, *trans*-3′-hydroxycotinine, and *trans*-3′-hydroxycotinine glucuronide. Nicotine and cotinine form *N*-quaternary glucuronides, whereas *trans*-3′-hydroxycontinine forms primarily an *O*-glucuronide. With chronic nicotine exposure, plasma cotinine levels are almost 15 times higher, whereas plasma *trans*-3′-hydroxycotinine levels are three to five times higher than plasma nicotine levels (Benowitz et al. 1990). Many animal species, including mice, dogs, rabbits, and monkeys, metabolize nicotine primarily to cotinine as humans do. In contrast, rats and guinea pigs form as much nicotine-*N*′-oxide as they do cotinine and 3′-hydroxycotinine [due to differences in the predominant cytochrome P450 (CYP) enzyme in these species; see below] and, as such, are not optimal models for investigating human nicotine metabolism.

Nicotine is extensively and rapidly metabolized by the liver and excreted to a small degree (on average about 5%), unchanged by the kidney. Urine excretion depends on pH and can vary as a percentage of total clearance, from less than 1% in alkaline urine to 20% or more in acidic urine. The average elimination half-life for plasma nicotine is 2 h in humans. With regular smoking, nicotine blood levels increase over 8 h, consistent with a rise to steady state over four half-lives ($t_{1/2}$). Even after overnight abstention, significant levels of nicotine are present in a smoker's blood in the morning, typically averaging 4–5 ng ml^{-1} (0.03 μM). When studied using highly sensitive assays, nicotine elimination in chronic smokers also appears to exhibit a very slow terminal $t_{1/2}$ of about 20 h, presumably reflecting slow release from deep tissue binding sites. The distribution $t_{1/2}$ of nicotine averages about 8 min (Benowitz et al. 1990).

Cytochrome P450 enzymes

The liver enzyme CYP2A6 is primarily responsible for the human metabolism of nicotine to cotinine and for the metabolism of cotinine to 3′-hydroxycotinine. CYP2B6 also may contribute to nicotine metabolism. *N*-Oxidation is mediated by the flavoprotein FMO3. Nicotine and cotinine glucuronidation appear to be mediated by UGT1A9, 1A4, and 2B7. A number of genetic polymorphisms in CYP2A6 that are associated with altered rates of human nicotine metabolism have been identified. Polymorphisms associated with absent CYP2A6 activity include the CYP2A6*4 deletion and CYP2A6*2 and *5 alleles; those associated with reduced activity include the CYP2A6*6, *7, *9, *10, *11 and *12 alleles. In contrast, a duplication polymorphism (CYP*1×2) has been reported to be associated with faster nicotine metabolism in humans. Polymorphisms in CYP2B6 have also been identified, but their impact on the rate of nicotine metabolism is undetermined, whereas identified polymorphisms of FMO3 do result in lower nicotine oxidation. While certain polymorphisms of CYP2A6 are associated with slower or faster nicotine

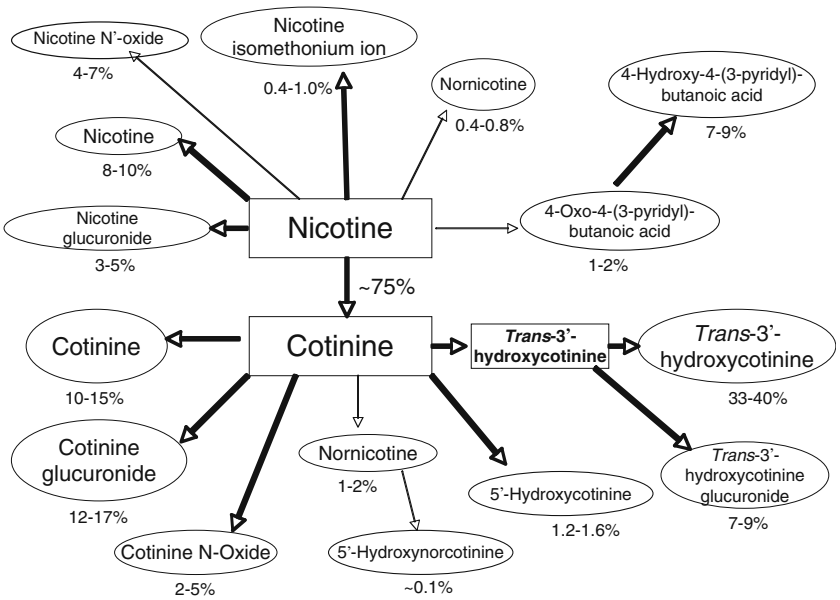

Fig. 1 Quantitative scheme of nicotine metabolism in humans. Percentages of nicotine and metabolites found in urine (Hukkanen et al. 2005)

metabolism, most of these are relatively uncommon in the general population. Even among individuals without identified polymorphisms, there is a wide variability in the rate of nicotine metabolism (Swan et al. 2005). This variation may be due to novel unidentified genetic variants or to other sources of variation, such as inducers or inhibitors. Investigators are referred to informative reviews (Malaiyandi et al. 2005; Miksys and Tyndale 2002) for additional details on cytochrome P450s, nicotine, and smoking.

Rate of metabolism

The rate of nicotine metabolism is determined by measuring blood levels at specific time intervals after administration of known doses of nicotine. The total clearance of nicotine (i.e., the amount of blood that has been cleared of the drug per interval of time) averages about 1,200 ml min^{-1} in humans, although chronic cigarette smoking itself slows clearance. Non-renal or metabolic clearance represents about 70% of liver blood flow (Hukkanen et al. 2005). As most nicotine is metabolized by the liver, about 70% of the drug is extracted from the blood as it passes through the liver. Because of this high degree of liver metabolism, there is considerable first-pass metabolism of nicotine before it enters the systemic circulation when dosed orally or i.p. Therefore, only about 30% of orally administered nicotine can be expected to reach the circulation, with the other 70% metabolized primarily to cotinine before reaching the blood stream. Thus, when conducting research where nicotine is dosed orally or i.p. (in either humans or animal models), first-pass metabolism and its effect on the relative dose exposure to nicotine, as well as the resultant levels of and exposure to cotinine, must be considered. In this regard, the cotinine levels measured from smoking or i.v. delivery are indicative of a substantially higher exposure of the brain and systemic circulation to nicotine compared to similar cotinine levels resulting from oral or i.p. delivery (Benowitz et al. 1990).

The metabolism in primates is comparable to that in humans (Seaton et al. 1991), but other animals metabolize nicotine more rapidly (Gorrod and Jenner 1975; Scheline 1978; Seaton and Vesell 1993). In addition, although the mouse CYP2A5 is a homolgue of human CYP2A6, in the rat, CYP1B1/2 is responsible for nicotine metabolism and CYP2A6 is inactive (Hammond et al. 1991; Nakayama et al. 1993). As indicated above, this makes the rat a less suitable model for investigations focusing on human nicotine metabolism. Plasma nicotine $t_{1/2}$ in rodents is generally shorter than in primates (45 min in the rat and 6–7 min in the mouse vs 2 h in humans and nonhuman primates), necessitating the use of higher daily doses of nicotine in rodent models to achieve the blood nicotine concentrations comparable to those seen in smokers. There also are significant strain differences in the rates of metabolism, even within species. Because of this variability of nicotine metabolism in experimental animals, the authors advise investigators to measure blood nicotine concentrations during steady-state dosing conditions in their specific species and strain to identify a dose achieving nicotine blood concentrations relevant to human exposure through cigarette smoking, smokeless tobacco, or nicotine replacement therapies.

Factors affecting metabolism

Age, sex, race, and disease states have been reported to affect nicotine metabolism in people. Total nicotine clearance is 23% slower in the elderly (age, 65–76 years) adult smokers compared to younger (age, 22–43 years) smokers (Molander et al. 2001), presumably due to a number of factors, such as lower CYP2A6-mediated metabolism, reduced liver blood flow, and slowed renal clearance. Women metabolize both nicotine and cotinine more rapidly than men do, with 13 and 26% higher clearance rates, respectively (Benowitz et al. 2004). In women who take oral contraceptives, the rates of nicotine and cotinine metabolism are 30 and 33% higher, respectively, compared to those who do not. In addition, during pregnancy, there is a marked acceleration in metabolism of both nicotine (60% increase) and cotinine (140%) compared to the postpartum levels (Dempsey et al. 2002). The levels of nicotine and cotinine in amniotic fluid are correlated with those in maternal blood, with average amniotic fluid-to-plasma ratios of 1.54 and 0.72, respectively (Luck and Nau 1984). There is also a high correlation between nicotine concentrations in breast milk and serum, although breast milk nicotine concentration is 2.5–2.9 times greater because it is relatively more acidic (pH 6.8–7.0) than serum (pH 7.4) and nicotine partitions into the more acidic medium (Dahlstrom et al. 1990; Luck et al. 1985). In addition, the high lipid content of breast milk and placenta tends to sequester nicotine and cotinine (Sastry et al. 1998).

Asians on average metabolize nicotine more slowly than Caucasians do, at least in part due to a high prevalence of the CYP2A6 alleles associated with reduced or absent enzyme activity (Benowitz et al. 2002; Schoedel et al. 2004). There are no significant differences in nicotine metabolism between Caucasians and Latinos; however, African Americans metabolize nicotine and cotinine more slowly than Caucasians do (Benowitz et al. 1999; Perez-Stable et al. 1998). In African Americans, both total and non-renal cotinine clearance are significantly lower (e.g., total clearance is 0.57 compared to 0.76 ml min^{-1} kg^{-1} in Caucasisans), although the genetic basis for this ethnic effect has not been established. This difference in metab-

olism results in higher levels of cotinine among African Americans for similar intakes of nicotine.

Chronic kidney disease is associated with reduced nicotine and cotinine renal clearance, as well as a 50% reduction in metabolic clearance of nicotine that may be attributable to inhibition of CYP2A6 activity or down-regulation of its expression in the liver (Molander et al. 2000). While many hepatic disorders, such as alcoholic liver disease (Sotaniemi et al. 1995) and hepatitis A (Pasanen et al. 1997), are associated with reduced CYP2A6-mediated metabolism, liver fluke parasitic infection actually induces it (Satarug et al. 1996). Certain known enzyme inducers, such as anti-convulsant drugs, rifampin, and oral contraceptives, also accelerate nicotine metabolism. CYP2A6 is inhibited by methoxsalen and tranylcypromine. The former has been shown to slow nicotine metabolism and to affect smoking behavior. In addition, smoking itself reduces the rate of nicotine metabolism, possibly via down-regulation of CYP2A6. This is an important consideration when comparing nicotine effects between smokers and non-smokers, as well as in some animal models of chronic vs acute exposure. Investigators are referred to additional resources (e.g., Benowitz 1998; Tyndale and Sellers 2001; Whiteaker et al. 2000a,b) and a recent comprehensive review (Hukkanen et al. 2005) for specific details and additional references on nicotine pharmacokinetics and pharmacodynamics.

Stress is an important environmental factor that may influence responses to nicotine, especially in animal studies (see "Acute nicotine treatment regimens" and "Repeated injection"), because it not only alters general metabolism but also effects several nicotine-responsive neurotransmitter systems (Balfour 1991; Benwell and Balfour 1982; Matta et al. 1993, 1998; Takahashi et al. 2000). As such, minimization of stress by pre-exposing the subjects to experimental procedures that may induce stress, such as administering saline injections before initiating the nicotine injections, is recommended.

Nicotinic cholinergic receptors

As the focus of this review is to provide insight for the judicious selection of a nicotine dose range and route of administration for in vivo studies, addressing specific details related to receptors and receptor subunit composition is outside its purview. The information below is only a brief overview and the interested reader is directed to excellent articles that focus on receptor-related issues (e.g., Dajas-Bailador and Wonnacott 2004; Gotti and Clementi 2004; Hogg et al. 2003; Jones and Sattelle 2004; Leonard and Bertrand 2001; Lindstrom 2003; Lukas et al. 1999; MacDermott et al. 1999; Mansvelder et al. 2006; McGehee and Role 1996; Stitzel et al. 2000; Whiteaker et al. 2000a).

The behavioral studies addressed in this review emphasize the effect of in vivo nicotine on nAChRs in the brain, as CNS activation is central to behavioral responses to nicotine. However, action at peripheral receptors (such as the neuromuscular junction) is always a consideration, especially with systemic administration of higher doses. Peripheral nicotinic receptors have a wide localization that includes muscle, neuroendocrine cells, peripheral blood leukocytes, and ganglia (Leonard and Bertrand 2001; MacDermott et al. 1999). In experimental paradigms using *C. elegans* ("In vivo nicotine dose selection in *C. elegans*") and zebrafish ["In vivo nicotine dose selection in zebrafish (*Danio rerio*)"], in vivo studies will also involve peripheral receptor activation because the common route of delivery is via the environmental medium.

All nAChRs, whether central or peripheral, are ligand-gated ion channels composed of five subunits. In the peripheral receptor at the neuromuscular junction, the pentameric channel consists of α1 (two), β1, δ, and ε (replacing the embryonic γ) subunits. In contrast, neurons express a different subset of receptor genes, coding for α and β subunits only. In mammalian tissues, these include α2 through α7 and β2 through β4. Two additional subunits, α9 and a10, are expressed principally in sensory, immune, and other tissues; the α8 appears only in the chicken. CNS nAChRs can be grouped functionally by affinity for nicotine. The low affinity (μM) nAChR is thought to be a homomer of five α7 subunits and can be localized either pre- or post-synaptically. The high affinity (nM) nAChRs, such as the predominant α4β2*, contain combinations of two α and three β subunits (Couturier et al. 1990a,b; McGehee and Role 1996). The multitude of subunit combinations, as well as their pre- or post-synaptic locations, account for the regional diversity of neuronal responses and subsequent behavioral consequences. Table 7, focusing on zebrafish, indicates some of the sequence homology between species for currently cloned nAChR subunits.

Blockade by nAChR antagonists is used to determine whether nicotine-evoked changes are indeed mediated via nAChR activation. Mecamylamine, a drug that blocks α2-α6* nicotinic receptors, crosses the blood–brain barrier and is generally used in doses ranging from 0.1 to 2.0 mg kg^{-1} in vivo, although its selectivity for nAChRs may be compromised at doses higher than 1 mg kg^{-1}. In contrast, hexamethonium (1.0 to 10 mg kg^{-1}), which cannot cross into the brain, acts only at peripheral nicotinic receptors. Dihydro-β-erythroidine (DHβE) is more selective for the widespread α4β2* nAChRs (Mansvelder et al. 2002), whereas α-conotoxin MII is the preferred antagonist for nAChRs containing α6 or α3 subunits (Kulak et al. 2002; Whiteaker et al. 2000a, b), central to the mesocorticolimbic reward pathway.

Receptor desensitization and resensitization

It is important to note that the relationship between the concentration of the drug in the blood and its neurochemical and neuroanatomical effects in the brain are complex because sustained exposure to nicotine can result in desensitization of the receptors through which the drug exerts its effects (Mansvelder et al. 2006; Pidoplichko et al. 1997; Wonnacott 1990). A slowly rising concentration of nicotine, therefore, may result in desensitization of some populations of nicotinic receptors without first depolarizing the cells on which they are located. Thus, some nicotine-stimulated neurotransmitter responses, such as increases in dopamine release in the nucleus accumbens (Balfour 2004; Fu et al. 2000a) or elevated norepinephrine secretion in the paraventricular nucleus (Fu et al. 2001; Sharp and Matta 1993), depend upon sufficient nicotine reaching the brain quickly. The cigarette is the most efficient drug delivery device for rapid delivery of a nicotine bolus to the brain, closely followed by an i.v. bolus injection into the jugular vein near the right atrium in animals (see "Intravenous nicotine self-administration"). Other modes of delivery, such as i.p., s.c., mini-osmopump, chewing tobacco, drinking water, or NRTs, do not deliver a nicotine bolus rapidly to the brain (Benowitz 1990). Despite this, nicotine delivered via these latter routes can lead to conditioned place preference (CPP) (Le Foll and Goldberg 2005a,b; Risinger and Oakes 1995), as well as dopamine-dependent locomotor activation (King et al. 2004; Sparks and Pauly 1999). Therefore, the rate and duration of administration can alter the balance of receptor desensitization and resensitization.

The dynamic complexity of the effects of nicotine on neuronal nAChRs also emphasizes the need to perform time course studies. These studies are best achieved using techniques such as in vivo microdialysis or i.v. sampling, in which serial samples are collected before and after nicotine administration. It is interesting that studies suggest that some responses, such as the increase in dopamine overflow in the nucleus accumbens dialysate, may persist for up to an hour after an acute nicotine injection (Benwell and Balfour 1992; Fu et al. 2000b; Imperato et al. 1986; Iyaniwura et al. 2001; Nisell et al. 1994). This is, perhaps, surprising because it has been shown that the receptors mediating this response are desensitized fairly rapidly and remain desensitized after an acute injection of nicotine (Balfour et al. 2000; Meyer et al. 2001; Olale et al. 1997; Pidoplichko et al. 1997). An enhanced affinity for nicotine in the desensitized state provides an alternative explanation and may underlie the development of tolerance. This is supported by the regionally specific upregulation of active (epibatidine-stimulated ^{86}Rb efflux) nAChRs with chronic nicotine exposure via osmopump (Nguyen et al. 2004). The neuronal nAChR binding of nicotine is also higher in smokers compared to non-smokers (Breese et al. 1997; Perry et al. 1999) and returns to non-smoking levels in former smokers (Breese et al. 1997). Additional studies, both in vivo and in vitro, are obviously necessary.

Summary

The pharmacokinetics and pharmacodynamics of nicotine are dependent on a number of variables, including rapidity of uptake (e.g., cigarette smoking), efficacy of metabolism (e.g., CYP2A6 in humans and mice vs CYP2B1/2 in rats), potential physiological effects of nicotine metabolites, and excretion (urine pH). Cytochrome p450 metabolism is an important consideration, given the correlation of CYP2A6 polymorphisms with individual susceptibility to nicotine between smokers of different races and ages, as well as the species difference in predominant CYP subtype. The rate at which nicotine reaches the CNS and the concentration achieved in specific regions are determinant factors in eliciting reward and dependence. The differences in rate and frequency of exposure may also significantly affect critical proteins such as CYP isoforms and nAChRs.

In vivo nicotine dose selection in humans

Introduction

The selection of the method of nicotine dosaging used in a given study should take into account a number of factors, including the hypothesis being tested and the practical limitations that might be imposed by the laboratory or other testing environment, including local smoking restrictions. This section describes methods and considerations involved in dosing with nicotine in non-tobacco form, usually done experimentally, compared to those pertaining to nicotine dosaging from smoking cigarettes, obviously done voluntarily. Unless specifically stated, all doses are reported as the free base.

Nicotine in non-tobacco form

Intranasal administration

Intranasal administration of nicotine rapidly delivers nicotine into the systemic circulation and the brain. Nasal spray nicotine primarily is absorbed through the nasal mucosa, not inhaled, and arterial levels begin to increase within 1–2 min because nicotine can pass easily through the nasal membrane. This route basically avoids hepatic "first-pass" elimination common to oral drugs ("Rate of metabolism"), although some nicotine from the spray is swallowed, is absorbed by the gastrointestinal (GI) tract, and subsequently does undergo first-pass metabolism. The biovailability of

nasal spray nicotine is approximately 50–75% (Benowitz et al. 1997; Johansson et al. 1991). After a standard 1.0-mg nicotine dose with Nicotrol NS (0.5 mg per spray × two sprays), the nicotine spray product approved by the Food and Drugs Administration, the arterial nicotine peaks at 10 ng ml^{-1} (0.06 μM) within 5 min, while venous levels peak at about 4 ng ml^{-1} (0.02 μM) after 25–20 min (Gourlay and Benowitz 1997). Arteriovenous equilibrium is reached at about 30 min post-administration. The variability in absorption between subjects can be substantial with the nasal spray, particularly relative to the patch, due to loss of spray into the nasopharynx (down the throat) or from sneezing or to individual differences in nasal absorption (Benowitz et al. 1997).

Oral administration

Although some early studies administered nicotine orally (Jarvik et al. 1970), this route has not been widely used because of first-pass liver metabolism (Benowitz et al. 1990; see also "Rate of metabolism") and because it was generally believed that attempts to overcome this first-pass loss with increasing doses would lead to aversive effects, such as bitter taste, nausea, or vomiting. However, the aversive taste of nicotine can be masked when delivered in fruit juice, coffee, or soda. In addition, it recently has been shown that orally administered nicotine can be well tolerated and yields plasma levels comparable to other forms of NRT in the range of 6–22 ng ml^{-1} (0.04–0.14 μM) (Westman et al. 2001).

Transdermal exposure

Nicotine may be administered transdermally (i.e., through the skin) with the use of skin patches. Several different patches are currently marketed and researchers should be aware of differences that may make one type more suitable for their purposes. One distinction is between patches that deliver nicotine continuously over a 24-h period (e.g., Nicoderm CQ, Habitrol, and ProStep) vs "daytime" (16 h) delivery (Nicotrol). A second important distinction is the rapidity with which peak nicotine levels are attained. Nicoderm delivers a rapid initial dose of nicotine, resulting in peak levels within 4 h of administration, whereas the other patches generally require 6–9 h to reach peak levels (Gore and Chien 1998). Therefore, in an acute laboratory administration paradigm, the appropriateness of one patch over another is dictated by the session duration and potential need to produce significant plasma nicotine concentrations within a certain time, as well as considerations about the effects of time varying vs steady nicotine levels. Having subjects apply patches the evening before a morning experimental session can result in relatively steady concentrations at the beginning of a session. Placebo skin patches, containing no nicotine and recently available (1-800-PATCHES, Salt Lake City, UT), are useful to control for expectancy effects.

In some studies, the design may entail administration of nicotine while subjects continue to smoke cigarettes (Rose and Behm 2004). Among the lay public and even among health professionals, it is widely believed that continuing to smoke while concurrently using NRT could lead to symptoms of nicotine overdose, including nausea, vomiting, or even death. However, studies of the effects of using NRT concurrently with cigarette smoking, as well as studies of high-dose NRT, using multiple skin patches or combinations of two or more forms of NRT, have found no evidence of serious toxic effects (Benowitz 2004; Benowitz et al. 1998; Schuurmans et al. 2004; Working Group for the Study of Transdermal Nicotine in Patients with Coronary Artery Disease 1994), indicating that smokers apparently have substantial tolerance to the adverse effects of nicotine.

Buccal administration

The first therapeutic use for oral nicotine was delivery through the buccal route with Nicotine Polacrilex (gum). While chewing a piece of nicotine gum, nicotine levels increase gradually over 15–30 min, and approximately half of the content of the gum is absorbed, i.e., 1 mg from "2 mg gum" and 2 mg from "4 mg gum" (Benowitz et al. 1990; Shiffman et al. 2002, 2004). The users must be instructed on proper use, such as intermittent chewing and "parking" the gum in or near the cheek, as well as avoiding acidic beverages that might interfere with the absorption of nicotine base. A second method of administering nicotine through the buccal mucosa is the nicotine inhaler (Nicotrol). The term "inhaler" is somewhat of a misnomer, in that nicotine in the vapor phase rapidly deposits in the mouth, and very little is inhaled into the lungs (Bergstrom et al. 1995). Thus, the pharmacokinetics resemble that of nicotine gum more than that of a cigarette. Given that the pharmacokinetics of the various buccal delivery systems are similar, the decision of which to employ in a given context may depend, in part, on other factors such as taste preferences and whether it is important for subjects to engage in a behavior that resembles puffing on a cigarette, such as would be achieved with the inhaler. However, the local irritating effects of nicotine, absence of the preferred sensory characteristics of cigarette smoke, and relatively slow nicotine absorption all make each of these products imperfect substitutes for cigarettes from a smoker's point of view and, therefore, introduce the challenge of maintaining compliance.

Intravenous administration

This route of nicotine administration has the potential advantage of mimicking most closely the pharmacokinetics of inhaled nicotine. It has often been supposed that pulmonary absorption of nicotine from inhaled cigarette smoke is much more rapid than by any other route (Russell and Feyerabend 1978), including i.v. administration. However, direct measurements of arterial nicotine concentrations during and after inhaling puffs from a cigarette compared to i.v. administration showed a similar time course (Fig. 2) (Rose et al. 1999).

Such similarity could be explained by a recent animal (rat) study demonstrating that the lung serves as an initial depot for nicotine and retards its entry into arterial circulation (Brewer et al. 2004). Therefore, rather than all of the nicotine inhaled in each puff being absorbed in a few seconds, evidence suggests it may require 30–60 s or longer for the nicotine to be absorbed, even though there is often an initial increase in levels after several seconds (although not in all smokers; see Fig. 3).

Many studies of i.v. self-administration of nicotine in humans have used a very rapid, high-dose bolus, although this may greatly overshoot the typical arterial and CNS nicotine concentrations achieved during smoking. The administration of doses ranging from 0.75 to 3.0 mg kg^{-1} in 10 s would be equivalent to smoking a typical cigarette in 10 s, instead of the usual 5–10 min (Harvey et al. 2004). These subjects often report intense aversive effects, as well as pleasurable stimulant effects not unlike those of cocaine (Jones et al. 1999; Harvey et al. 2004). In contrast, when nicotine is administered in puff-sized boli (0.1 mg kg^{-1} per injection), the subjective rewarding and aversive effects are minimal (Rose et al. 2003a–c). In addition, positive reinforcement, as evidenced by self-administration behavior, has been obtained using this more realistic dosing procedure (Rose et al. 2003a).

Nicotine in cigarette smoke and smoking models

Doses

A general issue common to studies with many of the nicotine delivery systems mentioned above is whether a fixed dose (or set of doses) should be administered to all the subjects in a study, as opposed to individualizing the dose. Rose et al. (2003c) developed a procedure for assessing nicotine intake per puff during a baseline ad lib smoking session and then, in subsequent experimental sessions, this dose was presented repeatedly using a puff volume control apparatus. While this procedure has the advantage of providing each smoker with the individual's comfortable level of nicotine, it has the drawback of precluding the assessment of a classic dose–response curve. The hypotheses under consideration will again dictate which of these factors is more important. In a study of physiological responsivity, obtaining a dose–response curve

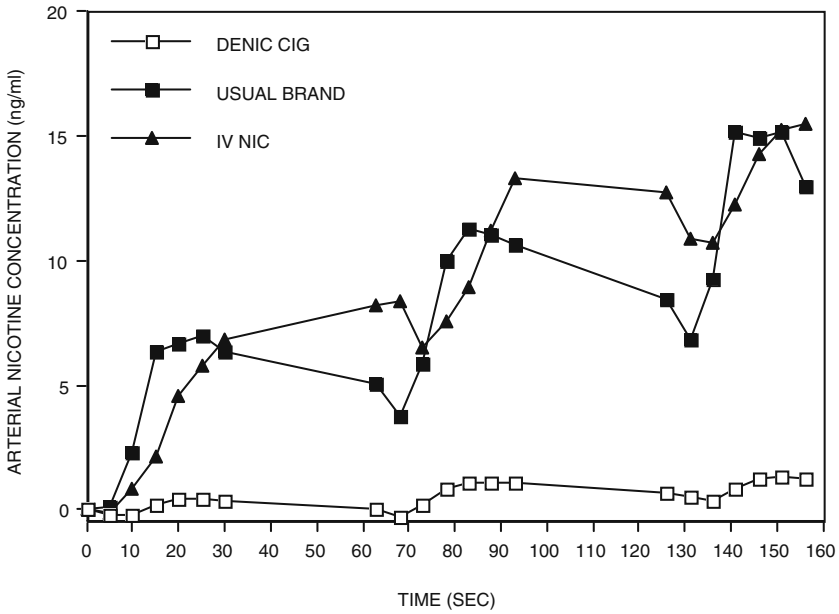

Fig. 2 Similarity in arterial nicotine concentration between cigarette smoking and i.v. injection. Arterial nicotine concentrations measured after three successive inhalations of puffs of nicotine-containing smoke (usual brand), denicotin-ized smoke, or i.v. administered nicotine (doses matched to usual-brand cigarettes; Rose et al. 1999)

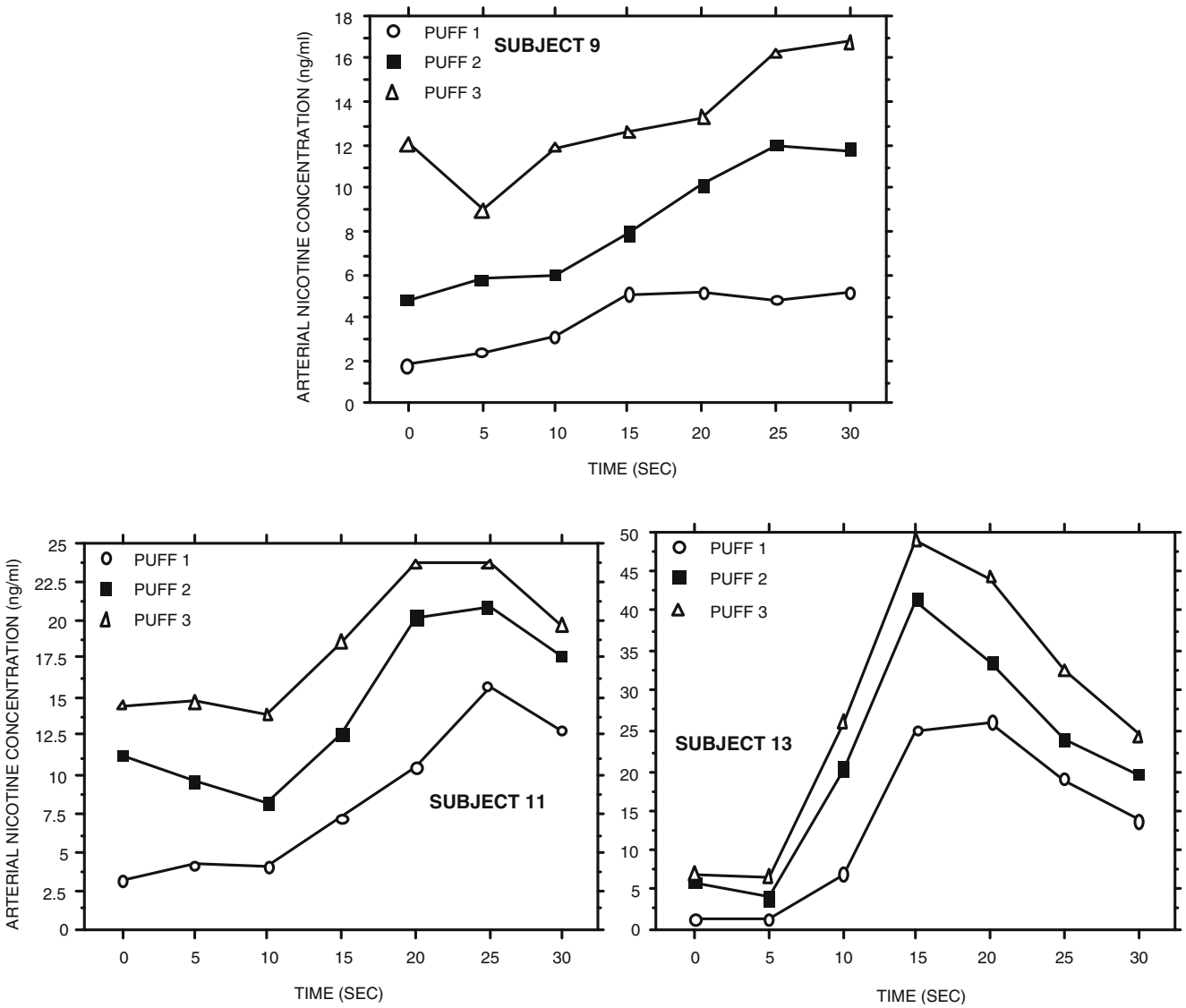

Fig. 3 Variability in arterial nicotine levels between smokers. Arterial nicotine concentrations measured during three successive puffs (usual cigarette brand), as shown by *three lines* within each panel, from three different smokers (*different panels*), showing inter-subject variability in response (Rose et al. 1999)

may be of paramount importance; in contrast, if the experimental design hinges on subjects deriving rewarding effects of smoking, then tailoring the dose may be preferable.

Variables affecting nicotine exposure via cigarette smoking

When cigarette smokers puff on a cigarette, they take varying numbers of puffs of different volumes and inhale to a varying extent (Fig. 3). Cigarette brands differ in nicotine content, presence of ventilation holes, and other construction factors. As such, both the manner in which the subject smokes and the brand of cigarette will determine the dose and rate of nicotine delivered.

Several methods of controlling puff volume and inhalation volume have been devised, which can effectively regulate the dose of nicotine inhaled (Gilbert et al. 1989; Levin et al. 1989; Pomerleau et al. 1989; Rose et al. 1985; Tashkin et al. 1991). It is recommended that one of these systems be used when studying the effects of inhaled nicotine, especially if the same smoker is being exposed to anything that may affect ad lib smoking, such as concurrent administration of the nicotinic receptor antagonist, mecamylamine.

It is also important to acknowledge that, aside from delivering nicotine, cigarette smoke contains many other substances and presents a rich set of sensory cues. Moreover, some evidence suggests that women smokers may be more influenced by these cues (Perkins et al. 2001). To attribute the effects being measured to nicotine, the incorporation of controls for at least some of these other

components are necessary. Several denicotinized or of reduced nicotine content tobacco cigarettes have been tested (Pickworth et al. 1999) and cigarettes containing tobacco with varying nicotine content are now commercially available (Quest brand). These cigarettes provide a means to control for many of the sensory- and cue-related aspects of smoking while still manipulating the nicotine dose.

Abstinence

When using any of the nicotine dosing systems described above for studies of the acute effects of nicotine, another issue is the length of time that the subjects should abstain from smoking or from using other nicotine delivery systems before dosing. The elimination half-life of nicotine averages about 2 h in humans (Benowitz et al. 1990); thus, after stopping smoking, at least 8 h of abstinence (overnight) may be required in order for nicotine levels and associated tolerance to decline to detect many of the physiological effects of nicotine. Standardizing smoking level is also likely to be important. Nicotine increases receptor binding in a dose-dependent manner (Marks et al. 1986a,b; Breese et al. 1997) and, perhaps, receptor sensitivity as well (Buisson and Bertrand 2002). As such, prolonged abstinence in a smoker may modulate nicotine responses, such as release of other neurotransmitters, in a manner different from a non-smoker (Leonard and Bertrand 2001). Furthermore, low concentrations of nicotine can remain in various tissue compartments, including the brain, for several days (see "Uptake and distribution").

Cognitive studies

In non-smoking adults, nicotine has been used in research studies of the cognitive effects of nicotine (Newhouse et al. 2004). Adults with attention deficit hyperactivity disorder (ADHD) (Levin et al. 1996b) and normal young adults (Levin et al. 1998), administered with a low-dose 7-mg patch for a single morning session, show significant improvement in attentional performance with only a few instances of short-term (a few hours) nausea, headache, and dizziness. Chronic nicotine skin patches have been used to improve attentiveness in people with mild to moderate Alzheimer's disease (White and Levin 1999), those with age-associated memory impairment (White and Levin 2004) as well as adults with ADHD (Levin et al. 2001) for 4 weeks at a time. In these studies, 5-mg patches for 16 h day^{-1} are used for the first week, followed by 2 weeks on a 10-mg patch 16 h day^{-1}, and finally a 5-mg patch in the last week. As in the acute studies, aside from transient nausea, headache, and dizziness in a few subjects, few adverse side effects have been reported; blood pressure and heart rate are not substantially altered and the 16-h patch can be used to limit sleep disturbance. Extensive reviews of nicotine's effect on mammalian cognition can be found in Levin and Rezvani (2002), Mansvelder et al. (2006), and Newhouse et al. (2004).

Individual differences can make it difficult to characterize inverted U-shaped response functions (a.k.a., a bell-shaped response curve) using the traditional fixed dose studies, because the variability frequently obscures sensitivity to some real effects in different subjects. This is particularly important for compromised populations such as the aged, in whom individual differences in kidney or liver function may alter pharmacokinetics and individual differences in the extent and character of neural decline may alter the pharmacodynamics of nicotine. A two-stage approach has been applied to human clinical testing for potential cognition-improving drugs, including nicotine. In this procedure, the optimal dose for each subject is identified in an initial dose study, followed by hypothesis testing with a dose range centered around this individual optimum (Buccafusco and Jackson 1991; Buccafusco et al. 1999). In this way, individual differences in pharmacokinetics and pharmacodynamics can be taken into account when characterizing the cognitive improvement of nicotine.

Summary

There are numerous nicotine exposure modalities in the human that can be utilized for experimental, therapeutic, or voluntary (i.e., smoking) purposes. Animal studies are necessary to identify and characterize the details of the underlying neurophysiology and neurochemistry, but it is the human condition that drives biomedical research. Therefore, the common factors of human nicotine use and abuse, such as routes of administration, duration of exposure, and dose (e.g., puff volume or transdermal patch concentration), sensory and environmental cues, and individual variability (i.e., genetic background, CYP2A6 activity), will comprise the original paradigms that subsequent animal studies attempt to model.

In vivo nicotine dose selection in nonhuman primates

Introduction

Studies in nonhuman primates offer the advantage that the genetic makeup, neuroanatom.y and behavioral characteristics of monkeys bear many resemblances to humans. They are amenable to imaging with positron emission tomography (PET), single-photon emission computed tomography (SPECT), and functional magnetic resonance imaging. Moreover, positive results in monkey behavioral models

translate reasonably well to humans, with a good level of human clinical predictability. However, simpler in vivo models such as rodents are generally less expensive, easier to acquire and house, and readily amenable to different experimental treatments, including surgeries. Therefore, initial work with such models, followed by studies in nonhuman primates, may offer the optimal approach to elucidate the role of the nicotinic cholinergic system and the effects of nicotinic receptor drugs in biological processes.

Nonhuman primate nAChRs

The transcripts for the α2 through α7 and β2 through β4 nAChR subunits have been identified and expressed in nonhuman primate neuronal tissues, with numerous receptor subtypes present in multiple brain regions (Cimino et al. 1992; Han et al. 2000, 2003; Quik 2004; Quik et al. 2000, 2001, 2005). However, the larger distribution of cortical α-bungarotoxin binding sites implicates a greater role of α7 receptors in these regions, compared to rodents (Han et al. 2003). These nAChRs are functional at both the cellular and whole animal level, e.g., robust nicotine-evoked dopamine release has been identified in monkey striatal synaptosomes (McCallum et al. 2005, 2006).

Acute nicotine treatment regimens

The primary mode of acute nicotine treatment is via intramuscular (i.m.) or i.v. injection, although nicotine has also been delivered by intracranial (i.c.) injection (Aizawa et al. 1999) (see Table 1). Nicotine is administered immediately prior (min) to testing. The i.m. route has been a preferred site of injection in protocols testing the effects of nicotinic receptor activation on attention, cognition, learning, and memory. These studies have been done in squirrel monkeys (*Saimiri sciureus*) with doses of nicotine from 0.1 to 1.0 mg kg^{-1} (32–325 μg kg^{-1}) (Hudzik and Wenger 1993) and in macaques (*Macaca mulatta*, *Macaca fascicularis*, and *Macaca nemestrina*) with doses generally ranging from 0.001 to 0.056 mg kg^{-1} (0.3–18 μg kg^{-1}) (Buccafusco and Jackson 1991; Buccafusco et al. 1995, 1999; Elrod et al. 1988; Katner et al. 2004; Prendergast et al. 1997; Terry et al. 1993; Witte et al. 1997). Similar endpoints have also been evaluated using an i.v. administered nicotine bolus of 0.1–1.0 mg kg^{-1} (32–325 μg kg^{-1}) in squirrel monkeys (*S. sciureus*) (Takada et al. 1989).

For PET studies, acute nicotine injection is also used, generally via bolus i.v. injection with or without chronic infusion. Baboons (*Papio*) and rhesus monkeys (*M. mulatta*) have been given nicotine doses ranging from 5 to 40 μg kg^{-1} (Ding et al. 2000; Fowler et al. 1998; Valette et al. 2003) or 0.01–0.065 mg kg^{-1} (3–20 μg kg^{-1}) (Marenco et al. 2004). These studies aim to mimic the doses achieved with smoking, where the nicotine content in cigarettes of 0.8 to 1.9 mg corresponds roughly to 10–30 μg kg^{-1} in the average human male. In addition, higher doses [up to 0.3 mg kg^{-1} (100 μg kg^{-1})] of nicotine over a 2-min period have been given, but these resulted in significant cardiovascular effects (Tsukada et al. 2002). Jugular infusion of 0.009–0.03 mg kg^{-1} [3–10 μg kg^{-1}] via a catheter has also been used (Goldberg and Spealman 1983), although this does invoke the complications associated with surgical implantations. Thus, multiple acute nicotine dosing protocols (i.m., i.v., and i.c.) have been used with doses generally in the low microgram per kilogram free base range, carefully tailored to optimize the effect of interest and minimize untoward side effects. Table 1, summarizing these acute studies, is meant to provide an overview rather than a comprehensive survey.

Repeated injection

Multiple injection paradigms have the advantage that dose and time of administration are well controlled. On a once or twice daily injection schedule, nicotine is also cleared entirely before the next injection, resulting in nAChR activation each day (Benwell and Balfour 1992). One drawback is that the injection process per se is stressful under some circumstances, which may affect a host of biological processes and on the interactions of nicotine and stress (see also "Rate of metabolism", "Acute nicotine treatment regimens", and "Repeated injection"). This mode of delivery has been used in studies to evaluate the effects of nicotine on metabolism in African green monkeys (*Chlorocebus aethiops*; Schoedel et al. 2003) using doses ranging from 50 to 300 μg kg^{-1} given twice daily for 18 days. The effect of nicotine on locomotor activity has been determined in hemiparkinsonian macaques (*M. nemestrina*; Domino et al. 1999) in whom nicotine attenuates the antiparkinsonian action of L-dopa at doses ranging from 32 to 320 μg kg^{-1} administered once daily for 6 days.

Chronic nicotine treatment regimens

Chronic nicotine exposure presents additional challenges compared to acute treatment as the dosing schedule must be reliably maintained over a prolonged time course without adversely stressing the animals. To this end, several regimens are available including treatment via the drinking water, s.c. infusion (osmotic minipump), i.v. self-administration, and exposure to cigarette smoke (Table 2).

Table 1 Representative studies using acute nicotine administration in nonhuman primates

Route	Species	Dose (mg kg^{-1}) [free base μg kg^{-1}]	Type of study	Reference
Injection (s.c.)	*Chlorocebus aethiops*	5–30 (bid 18 days)	Metabolism	Schoedel et al. 2003
Injection (i.m.)	*Macaca mulatta*, *Macaca nemestrima*, *Macaca fascicularis*	0.001–0.056 [0.3–1.8]	Attention, memory, cognition	Buccafusco and Jackson 1991; Buccafusco et al. 1995, 1999; Elrod et al. 1988; Katner et al. 2004; Prendergast et al. 1997; Terry et al. 1993, 1999; Witte et al. 1997
	Macaca nemestrina	32–320 × 6 days	Locomotor activity	Domino et al. 1999
	Saimiri sciureus	0.1–1.0 [32–325]	Cognition	Hudzik and Wenger 1993
Injection (i.v.)	*Saimiri sciureus*	0.1–1.0 [32–325]	Cognition	Takada et al. 1989
Bolus jugular infusion	*Saimiri sciureus*	0.01–0.03 [3–10]	Behavioural suppression	Goldberg and Spealman 1983
Intravenous bolus injection ± infusion	*Papio papio*, *Papio*	5–40	PET studies	Ding et al. 2000; Fowler et al. 1998; Valette et al. 2003
	Macaca mulatta	0.01–0.065 [3–20]		Marenco et al 2004
	Macaca mulatta	0.1–0.30 [32–100]		Tsukada et al. 2002
Intracranial microinjection	*Macaca fuscata*	0.4–2 μl of 1–100 mM	CNS electrophysiology	Aizawa et al. 1999

The doses are those reported in the original paper; if identified there as a bitartrate form, the doses in brackets are the conversions from bitartrate to free base nicotine, expressed as microgram per kilogram (μg kg^{-1})

Nicotine in drinking water

A relatively recent mode of nonhuman primate nicotine administration, initially used for rodents (see "Oral Administration", "Nicotine in drinking water" and "Nicotine in drinking water"), involves inclusion of the drug in the drinking water, usually with saccharin to mask the bitter taste. Nonhuman primates appear to prefer nicotine in an orange Tang drinking solution, indicating that Tang may better mask the taste of nicotine; this has not been tested

Table 2 Representative studies using chronic nicotine administration in nonhuman primates

Mode of administration	Species	Dose (mg kg^{-1}) [free base μg kg^{-1}]	Duration (days)	Type of study	Nicotine levels	Reference
Drinking water	*Saimiri sciureus*	0.050–0.65 mg ml^{-1} drinking solution	270	Neuroprotection	10–15 ng ml^{-1}	Quik, McCallum, Parameswaran (Fig. 4)
Osmotic minipumps	*Papio hamadryas*	1–2.0 day^{-1} [325–650 day^{-1}]	14–28	Biochemical measures/SPECT	27.3 ng ml^{-1}	Kassiou et al. 2001
	Macaca mulatta	1–3.0 day^{-1} [325–975 day^{-1}]	120	Respiration		Howell 1995
	Macaca mulatta (pregnant)	1–1.5 day^{-1} [325–488 day^{-1}]	135	Biochemical measures in newborns	13.8 ng ml^{-1} (amniotic fluid)	Grove et al. 2001; Sekhon et al. 1999
Intravenous self-administration	*Papio anubis*, *Macaca mulatta*	0.1–0.56 [0.3–180]		Nicotine reinforcement		Ator and Griffiths 1983; Slifer and Balster 1985
	Saimiri sciureus	0.1–3.0 [3–975]		Nicotine reinforcement		Goldberg et al. 1981; Spealman and Goldberg 1982; Spealman et al. 1981

The doses are those reported in the original paper; if identified there as a bitartrate form, the doses in brackets are the conversions from bitartrate to free base nicotine, expressed as microgram per kilogram (μg kg^{-1})

with rodents, however. This oral approach has the advantage that treatment is episodic, as it occurs only when animals drink, and is relatively stress-free. The caveat is that the precise control of nicotine intake and the maximal daily dose are limited by the normal pattern of fluid intake by each individual animal. Individual variability also determines the concentration of oral nicotine that will be tolerated before fluid consumption decreases and can lead to large variances of the mean consumption. Furthermore, any nicotine not absorbed immediately in the mouth is subject to first-pass metabolism upon swallowing and GI distribution (see "Rate of metabolism").

Oral nicotine intake may be particularly suitable for studies to evaluate long-term protection against chronic neurotoxic insults. For example, in a study published herein (Fig. 4), squirrel monkeys were given water containing 1% saccharin and, after 2 weeks of acclimatization, nicotine was added starting at a dose of 50 μg ml^{-1}. This was increased to 50 μg ml^{-1} $week^{-1}$ increments over a 3-month period to a final concentration of 650 μg ml^{-1} nicotine in saccharin, which was maintained for approximately 9 months. Fluid consumption was monitored and showed a trend for a decrease in the saccharin plus nicotine group compared to saccharin only similar to rodents, although this was not statistically significant (Fig. 4a). Some animals (<10%) initially refused to drink the nicotine-containing saccharin solution, consistent with individual differences in other experimental models. Food (pellets plus fruit) was also moistened with 25 ml of the nicotine/saccharin water. Nicotine and cotinine levels were determined throughout the treatment using high-performance liquid chromatography (HPLC), while the nicotine metabolite cotinine was measured using both HPLC and enzyme-linked immunosorbent assay (ELISA). At 650 μg ml^{-1} nicotine concentration, monkey plasma cotinine levels were in the 400-ng-ml^{-1} range with similar results using either ELISA or HPLC (Fig. 4c). The nicotine plasma values were 10–15 ng ml^{-1} (0.06–0.09 μM) (Fig. 4b), which are at the lower range of human smokers (10 to 50 ng ml^{-1}; see "Uptake and distribution").

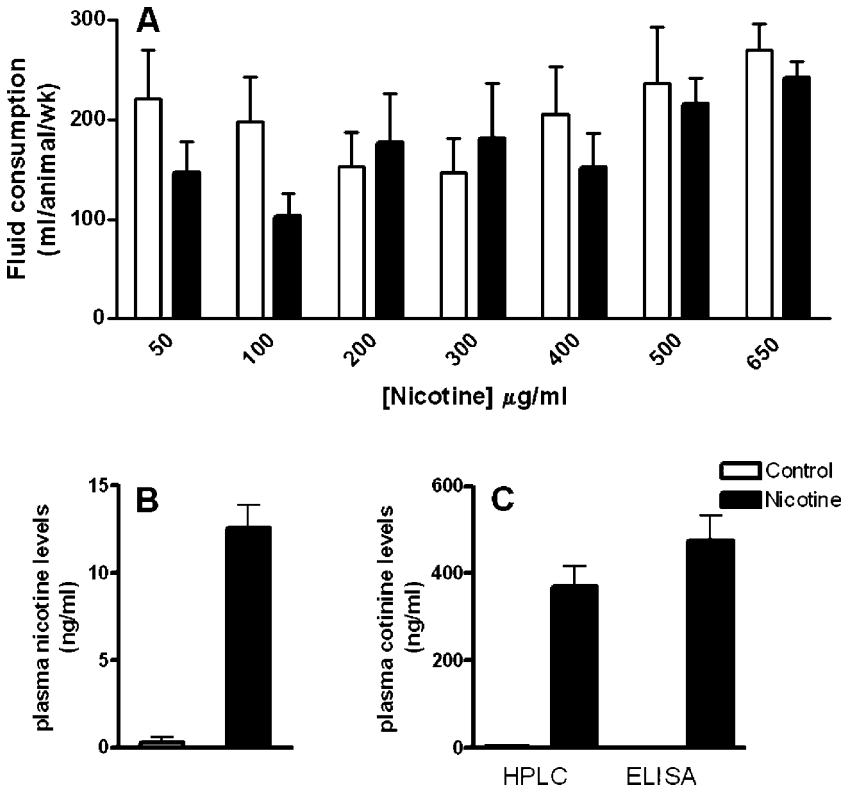

Fig. 4 Administration of nicotine to monkeys via the drinking water. Squirrel monkeys were given nicotine (free base) in drinking water containing 1% saccharin, starting at 50 μg ml^{-1} (0.3 μM) and then increased weekly in 50 μg ml^{-1} increments. **a** Weekly fluid consumption in animals given nicotine in saccharin compared to those receiving only saccharin. A similar volume of intake was observed in both groups. **b** Nicotine plasma levels in monkeys receiving both 650 μg ml^{-1} (4 μM) nicotine in drinking water and food moistened with nicotine. The plasma nicotine levels (10–15 ng ml^{-1}, 0.06–0.09 μM) achieved are similar to those seen in smokers (see "Uptake and distribution"). **c** Plasma levels of the nicotine metabolite cotinine in the same animals described in **b**. Note the similar cotinine levels whether measured using HPLC or by ELISA. The plasma cotinine levels (~400 ng ml^{-1}) are similar to those seen in smokers (15 times the plasma nicotine levels or 150–750 ng ml^{-1}; see "Cytochrome P450 enzymes"). Each value represents the mean±SEM, 9–13 monkeys (Quik, McCallum, and Parameswaran, published herein)

Osmotic minipump

The exposure to nicotine via osmotic minipump has the advantage of a slow chronic release over extended time periods, although it does involve a minor surgical procedure. This mode of delivery results in a chronic level of nicotine in the body in contrast to the pulsatile mode of delivery via smoking. Note that this may have functional significance, as chronic continuous nicotine exposure results in receptor desensitization, whereas receptor function fluctuates during episodic smoking, with periods of activation followed by desensitization, then resensitization (see "Receptor desensitization and resensitization"). Several chronic nicotine exposure paradigms utilizing minipumps in nonhuman primates have been reported. An infusion of 2 mg kg^{-1} day^{-1} (650 μg kg^{-1} day^{-1}) nicotine for 14 days in baboons yields plasma nicotine levels comparable to those in smokers (27.3 ng ml^{-1}, 0.17 μM). In that study, in vivo upregulation of nicotinic receptors was also demonstrated using SPECT (Kassiou et al. 2001). Chronic osmopump administration of nicotine at 1 mg kg^{-1} day^{-1} (325 μg kg^{-1} day^{-1}) for 4 weeks in rhesus monkeys has been used to assess the effects on ventilation (Howell 1995). To evaluate the effects of maternal smoking on fetal development, nicotine at 1–1.5 mg kg^{-1} day^{-1} (325 to 480 μg kg^{-1} day^{-1}) has been chronically infused into pregnant rhesus monkeys for 4 months (Grove et al. 2001; Sekhon et al. 1999).

Although osmotic minipumps provide a very convenient method for chronic nicotine delivery, the procedure has two inherent problems. First, the animals increase in weight over the course of the experiment, especially when lower doses of nicotine are used. Thus, the dose has to be calculated as a mean dose delivered over the course of the experiment, making it generally lower at the end of the study than at the beginning. Secondly, the stability of the drug is an issue. Nicotine solutions are commonly neutralized to pH 7.2–7.4 before administration and this should be done when giving nicotine as a s.c., i.p., or i.v. injection. However, nicotine solutions at neutral pH are relatively unstable and approximately 50% of the nicotine in a neutralized solution in a minipump will degrade over 10 days (Benwell and Balfour, unpublished observations). This clearly compromises data interpretation by contributing significantly to the change in the dose received by the animal over the course of the experiment. For this reason, the nicotine osmopump solutions should be acidic (approximately pH 4) and this is achieved when nicotine hydrogen tartrate (or bitartrate, rather than the free base) is dissolved in saline or water but not neutralized. Nicotine dissolved at this pH degrades more slowly, with <10% lost after 10 days (Benwell et al. 1995). When interpreting data using osmotic minipumps, it is again important to keep in mind the plasma concentration (not total dose) and the difference in pharmacokinetics between specific animal models and humans (see "Rate of metabolism").

Intravenous self-administration

Nicotine self-administration via i.v. infusion has been the focus of a number of studies investigating reinforcement in squirrel monkeys because of the advantage that episodic self-administration more closely resembles human smoking behavior (Goldberg and Spealman 1982; Goldberg et al. 1983; Henningfield and Goldberg 1983). Nicotine doses ranging from 0.01 to 3.0 mg kg^{-1} (3–975 μg kg^{-1}) have been self-administered through a chronically implanted jugular catheter. The optimal responses are observed at between 0.03 and 0.1 mg kg^{-1} (9.7–32 μg kg^{-1}), although the higher doses have been linked to side effects such as vomiting (Goldberg et al. 1981; Spealman and Goldberg 1982; Spealman et al. 1981). Nicotine self-administration via a chronic indwelling catheter also has been reported in baboons and rhesus monkeys, using similar doses of 0.001–0.56 mg kg^{-1} (0.3–180 μg kg^{-1}) (Ator and Griffiths 1983; Slifer and Balster 1985). See also specific experimental considerations in "Chronic nicotine treatment regimens".

Exposure to cigarette smoke

Studies examining the effects of smoking in nonhuman primates are at present quite limited, most likely due to difficulties of administration via this route (Ando and Yanagita 1981). PET studies have been done using baboons (*Papio papio*) trained to smoke cigarettes (Valette et al. 2003). Exposure to prenatal environmental tobacco smoke has also been investigated in pregnant rhesus (*M. mulatta*) monkeys, with a significant nAChR upregulation in the brains of the newborns after exposure to smoke for 6 h day^{-1} for 5 days per week for about 1 month (Slotkin et al. 2002). Further studies that investigate the effects of smoking are critical, as smoking is the primary human mode of nicotine delivery. In addition, although nicotine is one of the major determinants for addiction, the effects of other cigarette smoke components most likely also have significant biological actions and should be tested in nonhuman primate models. This knowledge is essential for a clear understanding of the effects of cigarette smoking vs nicotine alone.

Genetics and behavior

The strain-dependent measures are presented in Tables 1 and 2. A number of studies have shown that nicotine modulates reinforcement and a variety of behaviors including memory, learning and attention, and locomotor

activity (Buccafusco and Jackson 1991; Buccafusco et al. 1995; Domino et al. 1999; Goldberg et al. 1981; Schneider et al. 1998a,b). To demonstrate that nicotine-evoked behavioral changes are indeed mediated via nAChR activation, antagonists are tested for their ability to block a specific behavior. Mecamylamine, generally used in vivo because it crosses the blood–brain barrier, blocks nicotine self-administration (Goldberg et al. 1981; Spealman et al. 1981) and nicotine-mediated cognitive effects (Elrod et al. 1988; Katner et al. 2004) at doses ranging from 0.1 to 2.0 mg kg^{-1} (the selectivity of mecamylamine for nAChRs may be compromised at doses higher than 1 mg kg^{-1}). In contrast, such effects are not blocked by hexamethonium (1.0 to 10 mg kg^{-1}), which cannot cross into the brain and acts only at peripheral nicotinic receptors (Goldberg et al. 1981; Spealman et al. 1981). In addition, very low doses of mecamylamine have also been shown to exhibit agonist-like qualities in cognitive tasks (Terry et al. 1999).

Nicotine metabolites, such as cotinine, nornicotine and others ("Uptake and distribution"), may also contribute to the pharmacological profile of nicotine-induced behaviors. In delayed-matching-to-sample task accuracy by macaques, cotinine is only about 30-fold less potent than nicotine (Buccafusco and Terry 2003). Nornicotine (0.03 to 3.0 mg kg^{-1}, i.m.) produces a qualitatively similar effect to nicotine on schedule-controlled behavior in squirrel monkeys (Risner et al. 1985) and also affects the response rates and produces discriminative effects at doses similar to those of nicotine. Cotinine is less effective than nicotine in these tasks, with higher doses (~2,000×) required in eliciting similar responses (Takada et al. 1989). On the other hand, plasma cotinine levels are generally much higher than plasma nicotine levels. For instance, in the study reported herein, cotinine levels average 600 ng ml^{-1}, which is many times greater than the nicotine levels (10–15 ng ml^{-1}) (Fig. 4b,c, respectively). Moreover, cotinine persists in monkey plasma many hours after nicotine administration.

Summary

Despite the limitations associated with the use of nonhuman primates in research (cost, availability, handling, and surgery), the investigation of nicotine's actions and smoking in this model provides us with critical behavioral, physiological, and biochemical measures that closely model its effects in humans. As in humans, the effects of nicotine dosing in nonhuman primates vary according to the specific test procedure, as well as age, sex, and the individual subject under study (Buccafusco et al. 1999). An extrapolation of the results of published studies to novel experimental situations should consequently be done only after careful titration of dose. Finally, the route and the dose of nicotine administration are significant variables affecting blood and tissue levels and are important points to consider if the objective is to understand smoking behavior in humans.

In vivo nicotine dose selection in the rat

Introduction

Rat studies have provided a wealth of information on neurobiolgical mechanisms underlying systems relevant to humans, thereby providing an excellent experimental model for the effects of nicotine exposure, as well as translation of these preclinical findings to the human condition. In addition, the availability of well-characterized models of complex behaviors, such as motivated self-administration, locomotion, stress, drug discrimination, conditioned place preference, withdrawal, and cognition, make the rat amenable for testing a broad scope of hypotheses (see representative studies in Table 3). As such, more detail on the interaction of nicotine and behavioral paradigms is presented here, in comparison to other species.

Acute nicotine treatment regimens

In behavioral and neurophysiological experiments in rats and other animals, the effects of nicotine can exhibit an inverted U-shaped dose–response curve (a.k.a., a bell-shaped response curve), often with a peak response between 200 and 500 μg kg^{-1} for peripherally administered drug (Iyaniwura et al. 2001; Picciotto 2003). When nicotine is administered by daily i.p. or s.c. injection, peak brain nicotine levels are observed within approximately 15 min (Turner 1975) and are significantly lower than those achieved by i.v. injection or smoking a comparable dose (Benowitz and Jacob 1984). Therefore, the i.p. or s.c. doses frequently used are relatively large compared with those inhaled by cigarette smokers, and a single injection may result in blood nicotine levels significantly higher than those found in the venous blood of a smoker. However, due to the rapid metabolism of nicotine in rats compared to that in humans (see "Rate of metabolism"), the duration of elevated plasma nicotine concentrations is shorter ($t_{1/2}$=45 min vs 2 h). As such, with a daily injection schedule, nicotine is cleared entirely before the next injection, with one consequence being the activation of nAChRs by each dose (Benwell and Balfour 1992).

In experimental designs that minimized external stressors (also see "Repeated injection" for general discussion), acute nicotine injections (25–800 μg kg^{-1}, i.p.) to drug-naïve rats have been shown to increase plasma adrenocorticotropic hormone (ACTH) (Matta et al. 1987) and, subsequently, plasma corticosterone levels (Benwell and Balfour 1979; Caggiula et al. 1991). These findings are also

Table 3 Nicotine doses used in representative rat behavioral and neurochemical studies

Route	Doses	Response	Reference
Sc	200–800 μg kg^{-1} [65–260 μg kg^{-1}]	Locomotor activity: depression with acute injection, stimulation with daily injections	Clark and Kumar 1983; Morrison and Stephenson 1972; Stolerman et al. 1973
Sc	25 μg kg^{-1} [8.1 μg kg^{-1}]	Anxiogenic effects in the elevated plus-maze test	Cheeta et al. 2001
	100–450 μg kg^{-1} [32.5–146.3 μg kg^{-1}]	Anxiolytic effect in the social interaction test	Cheeta et al. 2001; File et al. 1998
Sc	100–400 μg kg^{-1}	Drug discrimination	Stolerman 1988; Stolerman et al. 1984
Sc	50–200 μg kg^{-1}	5-choice serial reaction time task	Hahn et al. 2002; Stolerman et al. 2000
Sc	300–500 μg kg^{-1}	Increased DA overflow in the shell of the nucleus accumbens in response to acute injection of nicotine	Cadoni and Di Chiara 2000; Iyaniwura et al. 2001; Nisell et al. 1997
Sc	100–500 μg kg^{-1}	Sensitization of the DA response to repeated nicotine administration in the core of the nucleus accumbens	Benwell and Balfour 1992; Cadoni and Di Chiara 2000; Iyaniwura et al. 2001
Sc	400–800 μg kg^{-1}	Increased norepinephrine overflow in the hippocampus	Benwell and Balfour 1997; Brazell et al. 1991; Mitchell 1993
Osmotic minipump	1–4 mg kg^{-1} d^{-1}	Desensitisation of locomotor activity; dopamine and nornorepinephrine release	Benwell and Balfour 1997; Benwell et al. 1995
Osmotic minipump	3–6.5 mg kg^{-1} d^{-1}	Nicotine dependence indicated by spontaneous withdrawal signs, increased reward thresholds, or reduced accumbal dopamine overflow	Epping-Jordan et al. 1998; Hildebrand et al. 1998; Malin et al. 1992, 1994; Skjei and Markou 2003
Iv	10–30 μg kg^{-1} and 45–135 μg kg^{-1}	Elevated plasma ACTH levels, cFos activation in noradrenergic and PVN CRH+ neurons	Fu et al. 1997; Matta et al. 1987, 1997; Valentine et al. 1996
	45–180 μg kg^{-1}	Increased norepinephrine release in amygdala, hippocampus, hypothalamic PVN	Fu et al. 1997, 1998a; Matta et al. 1995; Sharp and Matta 1993
	65–135 μg kg^{-1}	Increased dopamine release in nucleus accumbens	Fu et al. 2000a,b
Iv	3.8–90 μg kg^{-1} per injection	Operant self-administration	Brower et al. 2002; Corrigall and Coen 1989; Donny et al. 1995; LeSage et al. 2002; Shoaib et al. 1997; Valentine et al. 1997
Iv	30 μg kg^{-1} per injection	Cue dependency and environmental stimuli effects on self-administration	Caggiula et al. 2001, 2002
Iv	30 μg kg^{-1} per infusion	Nose poke	Belluzzi et al. 2005; Bespalov et al. 2005
Intracerebroventricular	0.25–5 μg per 500 nl	Increased PVN norepinephrine, elevated plasma ACTH	Matta et al. 1990, 1995
Intracerebral microinfusion	0.25–10 μg per 50 nl, 60 pmol, 8–24 nmol	Increased norepinephrine secretion in PVN and hippocampus, elevated plasma ACTH, CPP	Matta et al. 1993; Fu et al. 1999; Laviolette and van der Kooy 2003

The doses are those reported in the original paper; if identified there as a bitartrate form, the doses in brackets are the conversions from bitartrate to free base nicotine

relevant to addiction and locomotion studies because elevated plasma corticosterone can enhance the effects of drugs on dopamine release in the nucleus accumbens (Rouge-Pont et al. 1998). By way of contrast, repeated injections of 100–500 μg kg^{-1} nicotine reduces plasma ACTH and corticosterone levels, and a nicotine challenge dose (100–500 μg kg^{-1}) no longer elicits an increase of these stress-responsive hormones (Benwell and Balfour 1979; Sharp and Beyer 1986; Caggiula et al. 1991). A similar, albeit partial, habituation of the release of norepinephrine, a stress-related neurotransmitter, is also elicited by repeated injections of 500 μg kg^{-1}, i.p. (Sharp and Matta 1993) or 45–135 μg kg^{-1}, i.v. nicotine (Fu et al. 1998b). Microinjections of 0.25–5.0 μg per 500 nl nicotine into the

third or fourth cerebroventricles (Matta et al. 1990, 1995) or of 0.25–10 μg per 50 nl nicotine directly into brainstem noradrenergic centers (Matta et al. 1993) elicit norepinephrine release in the hypothalamic paraventricular nucleus (PVN) and subsequent ACTH release (Matta et al. 1998). In addition, nicotine has been shown to produce a bimodal stress-like response in the social interaction tests, in which anxiolytic effects are elicited by low doses (10–100 μg kg^{-1}, i.p.), whereas higher doses (500–1000 μg kg^{-1}, i.p.) have anxiogenic effects (File et al. 1998).

Chronic nicotine treatment regimens

To model more closely the chronic exposure experienced by habitual smokers, several approaches have been developed, each resulting in elevated blood nicotine concentration for some or all of the day. These include continuous nicotine delivery via s.c. osmotic minipumps (Benwell et al. 1995; Fung and Lau 1991, 1992), nicotine in drinking water (Maehler et al. 2000), and i.v. self-administration of nicotine (Corrigall and Coen 1989; DeNoble and Mele 2006; Donny et al. 1995; Valentine et al. 1997; Watkins et al. 1999). The plasma nicotine concentration maintained by each protocol may be critical to a specific hypothesis and should be measured whenever possible.

Nicotine in drinking water

Oral nicotine has been shown to activate taste pathways (Carstens et al. 2000; Dahl et al. 1997; Sudo et al. 2002) and these solutions are not particularly palatable, frequently requiring pre-training periods in which daily nicotine is combined with receding doses of saccharin over weeks of access, akin to the sucrose-fade method for alcohol (Samson et al. 1988) (also see "Nicotine in drinking water" and "Nicotine in drinking water"). When nicotine at 200 μg ml^{-1} (65 μg ml^{-1}) is included in the sole source of drinking water supplemented with 2% saccharin for 3 weeks, calcium-binding proteins in GABA neurons of the adolescent accumbens are upregulated (Liu et al. 2005). In a two-bottle free-choice method, rats will voluntarily consume nicotine at 0.003–0.006% concentrations in water, with gender-independent intake higher in younger than in older rats (Maehler et al. 2000). Investigators are reminded that plasma nicotine levels achieved by oral intake are significantly affected by first-pass liver metabolism (see "Rate of metabolism").

Subcutaneous osmotic minipump

The most commonly used method for chronic nicotine exposure is the s.c. osmotic minipump that delivers nicotine at a constant rate for up to 28 days (Alzet pumps, Durect, Cupertino, CA, USA; see caveats in "Osmotic minipump"). The doses of nicotine employed are commonly 1–4 mg kg^{-1} day^{-1}, with 1.0 mg kg^{-1} day^{-1} resulting in stable plasma nicotine levels of approximately 25 ng ml^{-1}, a concentration corresponding reasonably well with plasma levels in habitual smokers (Benwell et al. 1995). In many studies on withdrawal from chronic nicotine, rats are infused with approximately 3 mg kg^{-1} day^{-1} (Malin et al. 1992) and abrupt withdrawal (via removal of the minipump) results in somatic withdrawal signs (Epping-Jordan et al. 1998; Malin et al. 1992), as well as changes in neurotransmitter release in discrete brain sites (Carboni et al. 2000; Hildebrand et al. 1998, 1999; Hildebrand and Svensson 2000; Panagis et al. 2000) that are considered to underlie drug dependence processes. This dose is also high enough to lead to chronic desensitization of many nAchR subtypes in the brain, including those thought to mediate the effects of the drug on the mesolimbic dopamine neurons that are implicated in nicotine dependence (Benwell et al. 1995).

In gestational nicotine exposure studies, the use of the osmotic minipumps eliminates the fetal hypoxic consequence of uteroplacental vasoconstriction resulting from repeated acute injections (Seidler and Slotkin 1990). Doses of 2–6 mg kg^{-1} day^{-1} provide plasma levels approximately in the range of light (0.5–1 pack day^{-1}) and moderate (two packs a day) smokers (Lichtensteiger et al. 1988; Trauth et al. 2000). In utero exposure to 3 mg kg^{-1} day^{-1} results in a gender-dependent reduction in nicotine-stimulated dopamine release in the nucleus accumbens (Kane et al. 2003). However, exposure to 6 mg kg^{-1} day^{-1} or higher throughout gestation alters cholinergic and catecholaminergic neurodevelopment (Slotkin 1998), resulting in long-term gender-related behavioral deficits, such as hyperactivity, poor adaptation, increased adolescent anxiety, and cognitive deficits in adulthood (Vaglenova et al. 2004).

Intravenous nicotine self-administration

The first published report of i.v. self-administration of nicotine in the rat by Corrigall and Coen (1989) demonstrated that rats will self-administer nicotine if delivered as a rapidly injected i.v. bolus, approximating the nicotine bolus delivered by a cigarette, rather than as a slower infusion (10–20 s; also see Yanagita et al. 1995; Samaha and Robinson 2005; Shoaib 1996). In this limited-access model (1–2 h day^{-1}), rats pre-trained in bar press operant behavior will self-administer unit doses of 10–150 μg kg^{-1}, with a total daily nicotine intake ranging from 150 to 1,500 μg kg^{-1} (Chaudhri et al. 2005; Donny et al. 2000; Shoaib et al. 1997; Watkins et al. 1999). Longer periods of access (i.e., 6–23 h day^{-1}) have been used to model the

relatively unlimited daily nicotine intake in regular smokers (Brower et al. 2002; DeNoble and Mele 2006; Fu et al. 2001; Kenny and Markou 2006; LeSage et al. 2003; Paterson and Markou 2004; Valentine et al. 1997). Most of this motivated behavior occurs during the active (dark) phase of the diurnal cycle (Valentine et al. 1997) and total daily nicotine consumption ranges from 180 to 1380 μg kg^{-1} day^{-1}. In this model, tolerance to nicotine-stimulated release of norepinephrine, a stress-related neurotransmitter, develops (Fu et al. 2001). Both paradigms result in physical dependence, as measured by mecamylamine-precipitated somatic withdrawal signs and alterations in brain reward thresholds (see "Nicotine dependence and withdrawal"), although the duration of dependence may be greater after extended access self-administration (6 h; Kenny and Markou 2006; Paterson and Markou 2004). A number of variables affect i.v. self-administration, including feeding conditions (Donny et al. 1998), gender (Donny et al. 2000), prior exposure to nicotine (Adriani et al. 2003; Levin et al. 2003a), and age of onset of nicotine self-administration (Leslie et al. 2004). The critical factors of reinforcement schedule, strain, and environmental stimuli are addressed below.

Schedule of reinforcement Most models of nicotine self-administration employ a fixed ratio (FR) schedule of reinforcement that results in an inverted U-shaped curve (Corrigall and Coen 1989; Donny et al. 1995; Shoaib et al. 1997; Valentine et al. 1997), similar to that seen with other drugs of abuse. However, the nicotine curve tends to be flatter, with a narrow ascending limb of the curve that may reflect an averaging artifact between subjects exhibiting significant variations in behavior (E. Donny, unpublished observations). The response curve peaks at 15–30 μg kg^{-1} per injection nicotine, depending on strain, whereas higher doses generally decrease the rate of injections, regardless of duration of daily access. While this pattern indicates that animals titrate their behavior to attain an optimized or preferred overall level of exposure, this titration is, at best, incomplete and higher unit doses can elicit substantially greater daily nicotine intake in individual rats (Donny et al. 1995, 2000; Shoaib and Stolerman 1999; Shoaib et al. 1997; Valentine et al. 1997). A different pattern is seen in rats self-administering nicotine on a progressive ratio (PR) schedule of reinforcement. During a PR, the number of responses required to earn an infusion increases with each infusion self-administered, eventually resulting in a point at which the animal stops responding (i.e., "break point"). In contrast to self-administration behavior on an FR, the number of injections earned on a PR increases with dose. For example, nicotine doses from 20 to 90 μg kg^{-1} initially eliciting approximately eight to ten self-administrations on an FR5 over 2 h result in break points of approximately 120–1800, respectively, once the same rats are switched to a PR schedule (Donny et al. 1999). This PR behavior demonstrates a more persistent self-administration despite increasing behavioral costs and reflects motivation to work for the drug (Corrigall et al. 2001; Donny et al. 1999, 2000; Lanza et al. 2004; Paterson et al. 2004).

Strain There are significant strain-dependent differences in the acquisition of the behavior of i.v. self-administration of nicotine. In the limited-access model, Long–Evans, Sprague–Dawley, and Wistar rats acquire self-administration, but not the Fisher 344 or Lewis strains, at least not without pre-training and foraging behavior induced by food restriction (Chiamulera et al. 1996; Dworkin et al. 1993; Shoaib et al. 1997). At 30–60 μg kg^{-1} per injection, Long–Evans rats show a preference for the active over the inactive lever, whereas Sprague–Dawley rats self-administer doses as low as 15 μg kg^{-1} per injection (Shoaib et al. 1997). In the unlimited-access model, Holtzman, Lewis, and Wistar rats, but not the Fisher 344 strain, will self-administer 7.5–30 μg kg^{-1} nicotine per injection (Brower et al. 2002; Paterson and Markou 2004). However, a smaller percentage of Holtzman rats achieve individual self-administration criteria and responding is less robust (i.e., maintenance of self-administration when the nicotine dose is progressively reduced) compared to Lewis rats (32 vs 83%, respectively) (Brower et al. 2002).

Drug-associated stimuli Although nicotine alone supports self-administration, there are large differences in this behavior depending on whether the drug is paired with non-drug stimuli. Different stimulus conditions facilitate nicotine self-administration to differing degrees and the extent of this facilitation depends on the salience and reinforcing value of the non-drug stimulus (Caggiula et al. 2002; Donny et al. 2003; Le Foll and Goldberg 2005b). Figure 5 demonstrates that the dose–effect function is shifted to the left and upward in the presence of visual stimuli compared to when nicotine is not paired with the cues (i.e., onset of a stimulus light for 1 s and the turning off of the houselight for 60 s). A 30-μg-kg^{-1} nicotine dose, only marginally reinforcing in the absence of a visual stimulus, becomes a robust reinforcer when paired with the cues, with a two- to threefold increase in the maximal amount of nicotine self-administered (Caggiula et al. 2002; Chaudhri et al. 2005; Donny et al. 2000, 2003). It is surprising that, when large doses of nicotine are available (150 μg kg^{-1}), nicotine-associated cues add little to the behavior maintained by the drug alone (Chaudhri et al. 2005); the mechanism underlying this discrepancy has not yet been identified.

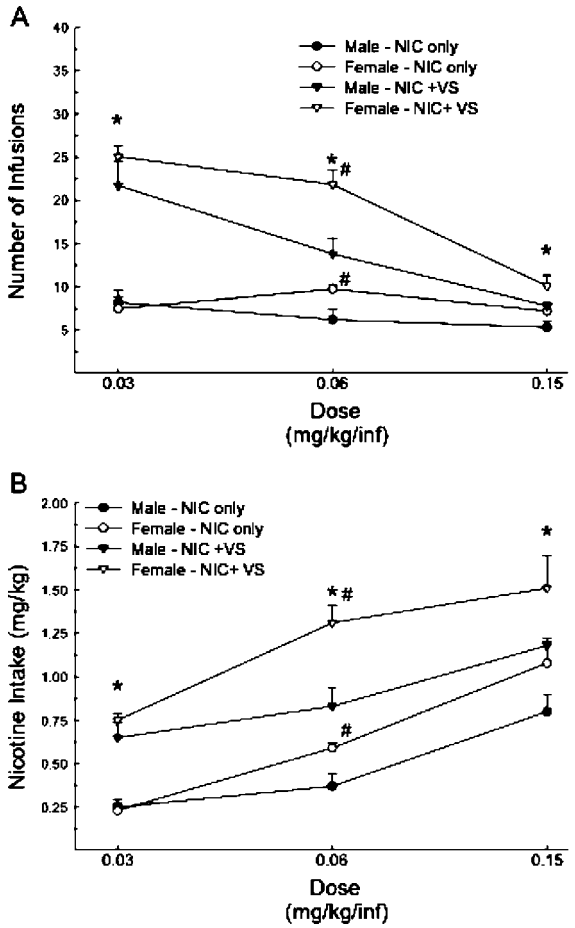

Fig. 5 Interaction of visual stimuli and gender on nicotine self-administration in a limited-access (1 h) model. Mean (±SEM) infusions (**a**) and nicotine intake (**b**) when nicotine was presented by itself (NIC only, *circles*) or paired with the onset of a cue light (1 s) and the turning off of the houselight (1 min) (NIC+VS, *triangles*) as a function of nicotine dose for male and female rats. Pairing nicotine with the visual stimuli (NIC + VS) earned more infusions compared to NIC only at the same dose for both genders (*$p<0.05$); *number symbol* indicates females had a higher nicotine intake compared to males at given dose and stimulus condition ($p<0.05$). (Chaudhri et al. 2005)

Nicotine-induced locomotor activity and sensitization

Nicotine has powerful but complex effects on the general activity of rats. The nature of these effects depends on dose, pre-exposure to both drug and testing apparatus, sex, age, and methods used to measure activity. Nicotine acutely produces a suppression of locomotor activity as measured by horizontal activity and rearing in an open field or Y-maze. This effect is dose dependent across a range of doses (20–100 μg kg^{-1}) when nicotine is injected s.c. or i.p. to drug-naive adult male rats who have not been habituated to the testing apparatus and, thus, would be expected to show high initial levels of exploratory behavior without the drug (e.g., Clark and Kumar 1983; Palmatier et al. 2003; Stolerman 1990). Both acute and chronic tolerance to this depressant effect develops rapidly and is replaced by sensitization, i.e., increased behavioral activation in response to subsequent injections (Clark and Kumar 1983; Stolerman et al. 1973). If animals are first habituated to the testing chamber, so that spontaneous locomotor behavior has largely subsided, a dose range of 100–500 μg kg^{-1} is likely to produce only locomotor activation that also increases with repeated daily exposure (Ksir 1994). The relationship between nicotine dose and other measures of activity, such as rearing (vertical activity) and stereotypy, is more complex and depends on factors such as route of administration, timing of measurement, strain, and sex (Faraday et al. 2003; Ksir 1994). Nicotine has similar effects on the locomotor activity of female rats (Kanyt et al. 1998), and while the range of doses is comparable between genders, details of the dose–response functions relating nicotine to activity can vary depending on interactions between strain, developmental stage, and the activity measurement used (Elliott et al. 2004; Faraday et al. 2003).

Nicotine drug discrimination

Doses

In the many reports on the discriminative stimulus properties of nicotine in the rat, nearly all have used s.c. administration and training doses ranging from 100 to 600 μg kg^{-1}. At a training dose of 400 μg kg^{-1}, nicotine produces a strong stimulus that is discriminated with at least 80% accuracy by the majority of rats after 30–40 training sessions (Chance et al. 1977; Pratt et al. 1983), whereas a 100-μg-kg^{-1} training dose requires nearly 60 sessions and is acquired with high accuracy by only 60–70% of rats (Stolerman et al. 1984). However, plasma nicotine levels associated with the 100 μg kg^{-1} training dose are approximately 35 ng ml^{-1}, which is closer to the typical plasma concentrations in inhaling cigarette smokers and may yield a stronger correlation of nicotine effects with high-affinity [^{3}H]-nicotine binding to nAChRs. The discriminative performance adequate for pharmacological investigation has not been seen with doses of 50 μg kg^{-1} or less, although few reports have used this range. In drug discrimination research, it is vital to be aware of the clear distinction between training doses of nicotine and the test doses used for defining dose–response relationships in previously trained rats. The larger the training dose, the quicker discrimination is acquired. Large training doses are also generally associated with steeper dose–response curves, and there may be changes in the specificity of the stimulus. Asymptotic accuracy increases with training dose, although the variation is small within the range of doses normally used. Rats trained with smaller doses of nicotine

are more able to detect small doses of the drug than those trained with larger doses are; as a consequence, the entire dose–response curve moves to the left when the training dose is decreased and the ED_{50} is lowered. For general reviews of drug discrimination methodology, see Järbe (1989) and Stolerman (1993).

Schedule of reinforcement

The schedule of reinforcement employed in drug discrimination research also influences the characteristics of the cue obtained (Overton 1979). Acquisition is quickest and performance is strongest with fixed ratio schedules, such as an FR10 in which the tenth response is reinforced. Variable interval (VI) schedules (e.g., VI over 60 s) produce appreciably slower acquisition but are typically associated with more finely graded generalization gradients, whereas FR schedules almost invariably produce quantal (all-or-none) generalization in individual animals (Stolerman 1989, 1991). Opinions differ as to which type of gradient is most appropriate (Colpaert 1991; Stolerman 1991). A tandem variable interval and fixed ratio schedule (Kuhn et al. 1974) has some of the advantages of both VI and FR schedules: acquisition is almost as fast as with FR schedules, and data can be obtained by means of lengthy extinction tests, which is not the case when simple FR schedules are used (Stolerman 1989). Food reinforcers have been used by most investigations into nicotine discrimination, with water and shock avoidance employed in the rest. The time between nicotine administration and behavioral testing also has a profound effect on quantitative aspects of nicotine discrimination (Stolerman and Garcha 1989). Most studies on nicotine discrimination establish two-choice nicotine vs vehicle discriminations, although other paradigms, such as drug vs drug, dose vs dose, and multi-choice discriminations, have been used (for a review, see Stolerman 1993). The training procedure selected has a powerful impact on the characteristics of the discrimination obtained and can influence the choice of dose. The role of rat strain, sex, and age has received little attention, although alcohol-preferring rats demonstrate a greater tendency to generalize from alcohol to nicotine than non-preferring rats do (McMillan et al. 1999).

Conditioned place preference

The reinforcing capacity of nicotine underlies the formation of the stimulus–reward associations leading to CPP; as such, stronger CPP is indicative of stronger reinforcement. A major difference between i.v. self-administration of nicotine and CPP is that self-administration directly assesses the reinforcing effects of nicotine, while CPP assesses the behavioral expression of conditioning processes. CPP has been elicited with s.c. doses of 0.06–1.4 mg kg^{-1} (Le Foll and Goldberg 2005a), although under some conditions conditioned place aversion can be produced by moderate (0.8 mg kg^{-1}; Jorenby et al. 1990) and high nicotine doses (1.2–2.0 mg kg^{-1}; Fudala et al. 1985; Le Foll and Goldberg 2005a). An important determinant of CPP is the relationship between initial (i.e., pre-test) preference and subsequent conditioning. The biased procedure, in which the drug is delivered in a chamber that was initially non-preferred, may be more effective for detecting nicotine-induced CPP than the unbiased procedure (Clark and Fibiger 1987; Le Foll and Goldberg 2005a; Shoaib et al. 1994). Other important considerations are the susceptibility of adolescents to develop preference compared to adults at the same dose (Belluzzi et al. 2004; Vastola et al. 2002) and strain differences, indicated by the increased development of CPP in Lewis rats compared to the Fisher 344 strain (Horan et al. 1997; Philibin et al. 2005). Both intracerebroventricular (i.c.v.) (1.2–18.5 nmol; Iwamoto 1990) and intra-ventral tegmental area infusions (8–24 nmol; Laviolette and van der Kooy 2003) also support CPP. The critical parameters for establishing CPP have been described recently (Le Foll and Goldberg 2005a,b).

Nicotine dependence and withdrawal

Withdrawal signs

Dependence on drugs of abuse, including nicotine, is often defined by the emergence of withdrawal symptoms upon abrupt cessation of drug administration. Nicotine withdrawal induced by cessation of tobacco smoking in humans is associated with an aversive withdrawal syndrome (Hughes et al. 1991; Shiffman and Jarvik 1976), the components of which are exhibited for 1–10 weeks (Hughes 1992) and are more severe in women than in men (Lynch et al. 2002). In rats, the cessation of investigator-administered nicotine alters a number of behavioral tasks, indicative of the presence of a withdrawal state (for a review, see Malin 2001). The exposure of rats to 9 mg kg^{-1} day^{-1} (3 mg kg^{-1} day^{-1}) for 7 days by osmotic minipump results in somatic withdrawal signs that represent the subtle nicotine-related subset of withdrawal signs on the opiate abstinence behavioral observation scale (Malin et al. 1992, 1997), the most commonly observed signs being ptosis, writhing, and gasping (Hildebrand et al. 1998; Watkins et al. 2000). These somatic signs can also be induced by the administration of a variety of nAchR antagonists (Epping-Jordan et al. 1998; Hildebrand et al. 1997–1999; Malin et al. 1998; Watkins et al. 2000). Furthermore, both the spontaneous and the precipitated nicotine withdrawal are associated with elevations in brain reward thresholds (Fig. 6) that reflect the affective depression-like aspects

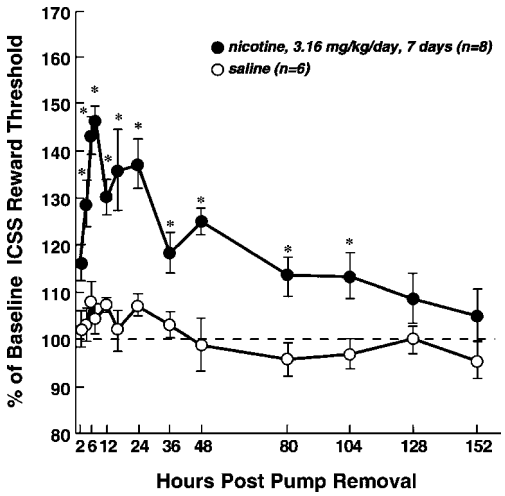

Fig. 6 Intracranial self-stimulation (ICSS) brain reward thresholds. ICSS reward thresholds in rats expressed as a percentage of the mean (±SEM) baseline reward threshold assessed before the implantation of the minipump. Brain reward thresholds during spontaneous withdrawal after termination of chronic administration of nicotine (3.16 mg kg^{-1} day^{-1}) (n=8) or saline (n=6). *p<0.05 for nicotine- vs saline-treated groups after minipump removal (adapted from Epping-Jordan et al. 1998)

of nicotine withdrawal (Epping-Jordan et al. 1998; Semenova and Markou 2003). It is interesting to note that the withdrawal from extended-access i.v. self-administration of nicotine (1 or 12 h day^{-1} for 20 days at 380 or 1,360 μg kg^{-1} day^{-1} nicotine, respectively) results in a protracted lowering of the reward threshold that lasts for at least 30 days (Kenny and Markou 2006). The discrepancy in nicotine-induced alterations in brain reward threshold between non-contingent high-dose nicotine and self-administered nicotine may be attributable to dose (Kenny and Markou 2005; Skjei and Markou 2003).

Modes of nicotine administration

The subcutaneous osmotic minipumps are the most commonly used means of inducing nicotine dependence in rats. A continuous exposure to 3.16 mg kg^{-1} day^{-1} nicotine for 6 days induces both the somatic and the affective aspects of nicotine withdrawal indicative of dependence that emerge approximately 6 h after cessation of nicotine administration and that persist for approximately 2–3 days (Malin et al. 1997, 1998; Skjei and Markou 2003). Increases in either dose or duration of exposure elicit small but consistent increases in both the magnitude and duration of the nicotine withdrawal syndrome (Skjei and Markou 2003). Nicotine dependence can also be induced via i.v. self-administration of nicotine in rats (Kenny and Markou 2006; Paterson and Markou 2004). Rats allowed to self-administer nicotine (30 μg kg^{-1} per injection) for 1 h day^{-1} (approximately 380 μg kg^{-1} day^{-1}) exhibit spontaneous nicotine withdrawal on day 25; with prolonged 6 h day^{-1} access (approximately 880 μg kg^{-1} day^{-1}), mecamylamine-precipitated nicotine withdrawal signs can be elicited for up to 2–4 weeks of abstinence.

Nicotine-induced effects on cognitive function

The effects of nicotine on cognitive function in rats are complex; many studies have demonstrated nicotine-induced cognitive improvement, while others have found no effect and some have even observed nicotine-induced impairment on cognitive tasks (Decker et al. 1997; Levin and Simon 1998). The typical inverted U-shaped dose–response relationship seen with all drugs eliciting cognitive improvement also varies considerably for nicotine, depending on which aspects are being tested (working vs reference memory), the demands of the task, and the extent of training; relatively high nicotine doses can actually impair performance. Further studies with nicotinic drugs specific for nicotinic receptor subtypes may offer promising leads for treating cognitive dysfunction (Levin and Rezvani 2002; Newhouse et al. 2004). The effects of nicotine on cognitive function are represented by the two cognitive assays described below.

Radial-arm maze win-shift procedure

In this procedure, nicotine has been shown to improve working memory function with a peak effective dose of 20 μg kg^{-1}, i.v. administered nicotine or with 5–12 mg kg^{-1} day^{-1} by osmotic minipump (Levin et al. 1993, 1994, 1996a; Levin and Torry 1996). This pro-cognitive effect of nicotine on working memory is specific because reference memory, which does not change throughout training, is unaffected by this same nicotine dose range. The 5 mg kg^{-1} day^{-1} nicotine dose does not improve performance in T-maze alternation, a task with considerable proactive interference (Levin et al. 1997). In some cases, the promnestic effect of nicotine can be eliminated or even reversed by altering neural systems that interact with the drug, such as acetylcholine, dopamine, serotonin, GABA, and glutamate (Wonnacott et al. 1989). For example, nicotine effects can be switched from improvement to impairment of working memory performance in the radial-arm maze by infusion of low doses of the *N*-methyl-D-aspartate glutamate antagonist dizocilpine that does not by itself impair working memory performance (Levin et al. 2003b). It is interesting to note that tolerance to the enhancing effects of chronic nicotine administration on radial-arm maze choice accuracy does not develop and a lasting improvement after withdrawal is observed, even when there is no training during the nicotine treatment period (Levin et al. 1992).

Five-choice serial reaction time task

In this model, nicotine 50–200 μg kg^{-1} improves choice accuracy and decreases response omissions when the task is run under specific conditions, such as unpredictable presentation of visual cues or imposition of an auditory distractor (Hahn et al. 2002; Mirza and Stolerman 1998). These nicotine-induced improvements in attentional accuracy are not attributable merely to an increasing motivation to perform the task because increasing food motivation results in a different array of changes on the task (Bizzaro and Stolerman 2003). In a lesion preparation of the basal forebrain, nicotine doses of 60 and 100 μg kg^{-1} (19.5 and 32.5 μg kg^{-1}, respectively) reverse the loss of choice accuracy, although higher or lower doses are ineffective (Muir et al. 1995).

Summary

The rat is currently the primary preclinical model for human nicotine exposure. Although nicotine metabolism is much faster ($t_{1/2}$=45 min compared to 2 h) and there is a difference in the major cytochrome P450 enzyme (CYP2B1/2 compared to CYP2A6), as well as subsequent metabolite levels, the differences in nAChRs and neurotransmitter mechanisms are relatively insignificant. The rat provides neurophysiological and behavioral correlates for nicotine dependence, tolerance, and withdrawal as well as insight into the interaction of nicotine with stress-responsive systems. Similar to human smokers, nicotine self-administration in the rat has been shown to depend on the critical factors of reinforcement schedule, genetic background (i.e., strain), and drug-associated environmental stimuli. The mechanisms underlying these and other nicotine-induced behaviors are the foci of most nicotine research because of the well-characterized neurophysiology and neurochemistry of the rat model.

In vivo nicotine dose selection in mice

Introduction

The laboratory mouse has been used to study the effects of nicotine behaviorally, physiologically, and biochemically, although this species has not been used as extensively as the laboratory rat. The availability of many genetically defined inbred strains with which to study variation in response to nicotine is a distinct advantage. With the advent of homologous recombination methodologies by which specific genes can be deleted or mutated, the mouse has assumed even more importance as an experimental model. Investigators are advised that significant physiological differences exist between rats and mice, making it inadvisable to utilize a direct application of rat protocols to mice without verification.

Acute nicotine exposure

In general, mice are less sensitive to the acute effects of nicotine than the rats are and, therefore, require a higher nicotine dose to achieve a similar response. For example, the ED_{50} dose for seizures in rats is 0.5–1.0 mg kg^{-1} (de Fiebre et al. 2002), while for mice it is 2–6 mg kg^{-1} depending on strain (Miner and Collins 1989). While the nicotine binding properties for high-affinity nAChR do not differ between rats and mice (Marks et al. 1986b), it is not clear whether pharmacokinetics can explain all of the observed species differences. For example, considerably higher brain levels of nicotine are required for comparable antinociception in mice compared to rats (Tripathi et al. 1982). The choice of nicotine dose and route of administration to be used in experiments with mice will be influenced not only by the specific behavioral or physiological response to be measured but also by mouse strain (see "Genetics and behavior" and Table 4).

Repeated injection

The extremely short half-life in the mouse (plasma and brain $t_{1/2}$=5.9–6.9 min after i.p. administration of nicotine at 1.0 mg kg^{-1}; Petersen et al. 1984) makes attainment of sustained nicotine levels via injection virtually impossible without frequent administration. Frequent, repeated nicotine injection unquestionably alters the subsequent responses of mice to a challenge dose of the drug, and this altered response certainly reflects a type of tolerance to the pharmacological effects of nicotine. It also is likely that the changes in behavioral responses observed after a regimen of multiple injections reflect stimulus–response associations (i.e., association of environmental cues with nicotine) and a stress–nicotine interaction (see also "Acute nicotine treatment regimens"). The development of tolerance to nicotine effects on Y-maze activity, heart rate, and body temperature after an injection of 2 mg kg^{-1} nicotine three times a day could probably indeed be attributed to an altered stress response, as indicated by elevated levels of corticosterone, rather than a specific nicotine-induced adaptation, as the density of nicotinic receptor binding sites measured with [^{3}H] nicotine and [^{125}I]α-bungarotoxin is unchanged (Pauly et al. 1992). The role of adrenal hormones in this manifestation of tolerance observed after repeated injections is supported by the elimination of tolerance after adrenalectomy (Grun et al. 1992) and the induction of tolerance after implantation of corticosterone pellets (Pauly et al. 1990). Tolerance observed with chronic exposure to corticosterone seems to differ from that

Table 4 Comparison of effective nicotine doses for effects on responses in 19 inbred mouse strains

Mouse strain	Respiration rate	Acoustic startle response	Heart rate	Y-maze crosses	Y-maze rears	Body temperature	Clonic seizure
A/J/Ibg	0.78±0.09	−1.67±0.61	0.82±0.13	0.80±0.31	0.41±0.21	0.55±0.06	3.12±0.13
AKR/J	1.48±0.09	−0.23±0.46	1.60±0.25	1.42±0.31	1.26±0.27	1.37±0.20	4.95±0.34
BALB/CbyJ	0.67±0.17	+2.04±1.48	0.95±0.33	1.06±0.06	0.97±0.20	0.92±0.17	3.65±0.14
BUB/BnJ	1.29±0.23	+2.27±1.75	1.48±0.24	1.89±0.33	1.48±0.24	2.53±0.08	4.52±0.06
CBA/J	0.73±0.31	+2.66±0.94	1.41±0.19	1.43±0.21	1.41±0.21	1.56±0.36	3.63±0.09
C3H/2Ibg	1.10±1.14	+3.70±0.73	1.25±0.24	1.78±0.33	1.50±0.10	1.32±0.09	3.13±0.08
C57BL/6J	0.95±0.19	−1.77±1.05	0.90±0.23	0.51±0.18	0.45±0.18	0.80±0.16	5.30±0.26
C57BL/10J	1.14±0.17	+0.73±1.05	1.12±0.12	0.49±0.21	0.37±0.27	0.61±0.21	3.55±0.40
C57BR/cdJ	0.43±0.24	−0.76±0.22	1.40±0.20	1.07±0.13	0.92±0.11	1.59±0.32	4.62±0.01
C57L/J	0.97±0.47	−0.10±0.06	1.36±0.40	1.17±0.27	0.80±0.12	1.20±0.11	4.99±0.05
C58/J	2.66±0.41	+0.49±0.94	1.28±0.48	1.82±0.08	1.54±0.22	2.07±0.06	5.89±0.19
DBA/1J	1.49±0.11	−0.10±1.32	0.94±0.24	0.93±0.31	0.94±0.42	1.02±0.26	6.16±0.02
DBA/2J	1.25±0.11	−0.80±0.87	0.94±0.24	0.97±0.31	0.80±0.06	0.89±0.19	5.21±0.12
LP/J	0.75±0.30	−1.18±0.54	1.79±0.35	1.04±0.34	0.95±0.26	1.30±0.15	4.50±0.04
P/J	0.77±0.23	−0.20±2.30	1.34±0.23	1.25±0.17	0.96±0.15	1.10±0.12	4.30±0.02
RIIIS/J	0.93±0.43	+1.44±0.41	1.98±0.79	1.62±0.17	1.46±0.17	1.19±0.17	3.65±0.14
SJL/J	1.00±0.14	+0.11±0.95	2.03±0.71	1.32±0.24	1.18±0.22	1.23±0.09	4.73±0.24
ST/bJ	0.41±0.04	+4.52±0.94	0.98±0.18	0.93±.0.21	0.64±0.27	1.47±0.23	2.34±0.09
SWR/J	1.19±0.25	−0.28±0.46	2.19±0.45	1.42±0.49	1.19±0.36	1.18±0.20	4.48±0.12

The values for respiration rate ($ED_{260\ breaths/min}$), acoustic startle response (slope of the dose–response curve), heart rate ($ED_{-100\ beats/min}$), Y-maze crosses (ED_{50}), Y-maze rears (ED_{50}), and body temperature ($ED_{-2°C}$) have been obtained from Marks et al. (1989). The values for clonic seizures (ED_{50}) have been obtained from Miner and Collins (1989). The units for all values are milligrams of nicotine (free base) per kilogram of mouse BWt, except for the acoustic startle response values which are slopes

observed after chronic nicotine infusion (Robinson et al. 1996a), suggesting that the adaptation to the effects of nicotine resulting from these two treatment procedures involves different neuroadaptive mechanisms. Therefore, unless a very specific experimental goal requires the repeated injection of nicotine to evaluate a specific response (e.g., the role of repeated stress or environmental cues on tolerance development), chronic administration of nicotine to mice by frequent injection as a model for human smoking may not be generalizable as a model for tolerance. As such, the results of such studies must be interpreted with caution.

In contrast, the repeated but intermittent injections of nicotine are required for several specific behavioral tasks including, for example, the development of nicotine place preference, nicotine-induced locomotor effects or antinociception, and learning of a drug discrimination response (Damaj 2005; King et al. 2004; Naylor et al. 2005; Picciotto 2003; Stolerman et al. 1999). The intermittent injection of nicotine used in these paradigms is fundamentally different than the more frequent injection design used in the studies discussed above. Several methods for chronic nicotine treatment that avoid repeated handling of the animals are available, resulting in tolerance to the effects of nicotine, and have been shown to increase nicotinic binding sites (see below). Therefore, it is the underlying hypothesis of the experiments to be conducted that will identify which method should be used.

Chronic nicotine exposure

Nicotine in drinking water

A simple method for chronic nicotine exposure is to supply nicotine in the drinking water (Rowell et al. 1983; Sparks and Pauly 1999). When given a choice of water or a nicotine solution, mice will drink nicotine, though rarely showing an actual preference for nicotine (flavored or unflavored) over water (Meliska et al. 1995; Robinson et al. 1996b). Consumption varies with sex and age, with adolescent female mice drinking higher concentrations of nicotine and consuming more (Meliska et al. 1995; Klein et al. 2004). The amount of nicotine consumed is also strain dependent, varying from 4 mg kg^{-1} day^{-1} for C3H mice to as much as 12 mg kg^{-1} day^{-1} for C57Bl/6 mice (Robinson et al. 1996b). The nicotine concentration at which aversion is seen is strain dependent, varying from an IC_{50} value of ~40 μg to 100 μg ml^{-1} (Robinson et al. 1996b), although some strains will tolerate nicotine concentrations as high as 200–500 μg ml^{-1} in saccharin-flavored water without adversely affecting daily fluid intake (M. Marks, unpublished results). This method has been shown to elicit a concentration-dependent increase in plasma cotinine and

the development of tolerance to a nicotine challenge dose 1.0 mg kg^{-1} measured with open-field activity and body temperature, as well as a dose-dependent increase in the density of receptor binding sites in C57BL/6 mice (Sparks and Pauly 1999). Chronic oral administration has also been used successfully to demonstrate dependence (withdrawal) and tolerance (200 μg ml^{-1} for 14–28 days and 50–200 μg ml^{-1} for 42 days, respectively; Grabus et al. 2005), as well as alteration in signaling pathways (Brunzell et al. 2003). It is especially attractive if long-term (weeks to months) nicotine exposure is desired, although the precise control of dose and timing of exposure are not feasible (see "Nicotine in drinking water"). For the investigator aware of these caveats, however, oral administration of nicotine is a very attractive method for simple, long-term nicotine exposure and the induction of dependence in mice.

Osmotic minipumps

Osmotic minipump delivery is well suited for the continuous administration of nicotine to attain steady-state blood levels in mice (Damaj et al. 2003), especially given their high rate of nicotine metabolism. A nicotine dose at 24 mg kg^{-1} g^{-1} for 14 days elicits antinociception (a measure of the induction of nicotine tolerance; Damaj 2000) as well as strain-specific severity of withdrawal signs (C57/BL are more sensitive than 129/SvEv; Damaj et al. 2003). Strain-dependent effects also have been demonstrated on auditory-evoked potentials after 2 weeks of exposure to nicotine at 4.2 mg kg^{-1} (57BL/6J and DBA/2Hsd; Metzger et al. 2006). Finally, withdrawal after exposure to nicotine at 6.3 mg kg^{-1} day^{-1} for 14 days impairs contextual fear conditioning in C57J/Bl6 mice (Davis et al. 2005). Concerns about cessation of treatment necessitating stressful surgical removal and alterations in dose per body weight over time are addressed in "Osmotic minipump".

Intravenous infusion

First, a few caveats. Mice are relatively resistant to nicotine, thereby requiring higher doses than those used to elicit a similar response in other species, including rat. Their rapid nicotine metabolism necessitates the administration of relatively high and frequent doses to attain nicotine plasma levels comparable to those in other species. The differences in blood plasma levels as a function of treatment dose for continuous administration of nicotine to rats using osmotic minipumps (Rowell and Li 1997) and mice by i.v. infusion (Marks et al. 2004) are presented in Fig. 7, illustrating that the hourly nicotine dose required to achieve plasma levels comparable to those of the rat is approximately tenfold higher in mice.

In addition, mice can be stressed by frequent injection of nicotine, as demonstrated by the high levels of corticosterone after repeated injection (Pauly et al. 1992) (see also

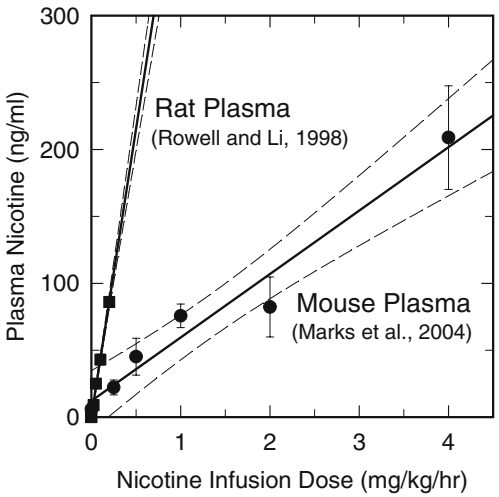

Fig. 7 Comparison of nicotine levels in rat and mouse plasma after chronic nicotine infusion. Due to the differences in metabolism between the two species, higher doses on an *hourly* basis are required in the mouse to approximate chronic plasma nicotine levels in the rat (modified from Rowell and Li 1997 and Marks et al. 2004)

"Rate of metabolism", "Acute nicotine treatment regimens", and "Repeated injection"). In contrast to rats, some strains of mice respond to frequent handling by becoming agitated rather than calm (Grabus et al. 2005; MJ Marks, unpublished observations). Therefore, choosing a method for chronic nicotine treatment must take into account each of these crucial aspects of mouse behavior and physiology.

The i.v. administration of nicotine to mice by infusion through a jugular cannula has been used extensively. Tolerance development is time (Marks et al. 1985) and dose dependent (Marks et al. 1986b) as are changes in the density of nicotinic binding sites. This method has been used to evaluate the role of genotype in tolerance development (Marks et al. 1991). In addition, nicotine dose and the kinetics of administration can be readily adjusted (Marks et al. 1987) with precise control of both the timing of treatment initiation and of cessation. The method requires surgical implantation of an indwelling jugular catheter, the availability of specialized cages and syringe pumps for each animal, and the possible confounder that tethering would constitute a mild stress. The method is very useful for (1) continuous or intermittent, experimenter-controlled drug exposure, (2) precise control of the timing of treatment initiation or cessation, and (3) changing doses during the experiment.

Nicotine self-administration

Nicotine self-administration has not been studied as extensively in mice as in rats because, in general, it is difficult to achieve and maintain self-administration in this species. Mice will self-administer nicotine under a number of conditions, though not avidly. The process of i.v. self-administration of nicotine in mice has been successfully

used by a number of investigators. One method involves delivery of nicotine through a tail vein cannula with the mouse nose-poking on an FR1 schedule with no time-out period. Nicotine self-administration can be supported by a narrow range of concentrations (0.01–0.05 mg kg^{-1} per injection), while lower or higher concentrations are not reinforcing (Blokhina et al. 2005; Martellota et al. 1995; Rasmussen and Swedberg 1998; Semenova et al. 2003). In contrast, a dose of 0.1 mg kg^{-1} is reinforcing in C57Bl/6J mice with jugular catheter administration and lever pressing as the required operant response (Stolerman et al. 1999). This difference may reflect a potential contribution of stress-related effects due to the restraint required for tail vein administration. Intracerebral injection directly into the ventral tegmental area, triggered by a Y-maze photocell beam break, has recently been shown to support nicotine self-administration in C57Bl/6J mice at 0.1 ng per injection (0.03 ng per injection) (Maskos et al. 2005). This concentration via jugular catheter also maintains nose-poke responding in C57Bl/6 mice initially pre-trained to self-administer cocaine (Picciotto et al. 1998). In both studies, self-administration was not maintained by saline and nicotine is not self-administered by mice with a null mutation for the nAChR β2 subunit.

Genetics and behavior

Genotype

Mouse genotype markedly influences the effect(s) of nicotine, including responses such as locomotion and body temperature (Marks et al. 1989) or clonic seizures (Miner and Collins 1989). As summarized in Table 4, EC_{50} values between inbred strains differ nearly fourfold for Y-maze crosses, 4.6-fold for body temperature, and greater than 2.5-fold for seizure sensitivity. Genotype also influences the pattern of response. For example, while three mouse strains (DBA/2, BALB/cBy, and C57BL/6) show reduced activity in an open-field arena after acute i.p. injection of nicotine doses of 0.5 mg kg^{-1} or higher, C3H mice display locomotor activation after administration of similar doses (0.5 to 1.0 mg kg^{-1}) and depression at a higher dose of 1.5 mg kg^{-1} (Marks et al. 1983).

Behavioral responses measured

The nicotine dose used to elicit an effect in a specific test can vary markedly based on the measurement under investigation. For example, EC_{50} values for male ICR mice after a s.c. injection of nicotine ranges from 0.5 mg kg^{-1} [eliciting hypomotility, antinociception (hot plate), and anxiolysis (plus maze)] to 1 mg kg^{-1} [antinociception (tail flick) and hypothermia] and up to 5 mg kg^{-1} (seizure induction) (Damaj 2001). In contrast, doses as low as 0.1 mg kg^{-1} elicit marked effects on other anxiolytic properties of nicotine, such as avoidance behavior (Brioni et al. 1993). Some responses to nicotine increase with increasing dose, such as body temperature, nociception, and seizure induction. Other effects of in vivo nicotine, particularly complex responses, result in distinct inverted U-shaped dose–response curves. The examples are conditioned place preference (Risinger and Oakes 1995), i.v. self-administration of nicotine (Martellotta et al. 1995; Semenova et al. 2003), and anxiolytic responses, including elevated plus maze (Brioni et al. 1993), mirrored chamber (Cao et al. 1993), and fear conditioning (Gould and Higgins 2003). Any behavior that reflects a balance between reward and aversion is likely to show a complex dose–response relationship and this balance frequently occurs in mice over a very narrow dose range. For example, CPP is observed after treatment with 0.5 mg kg^{-1} nicotine, but not with 0.25 or 1.0 mg kg^{-1} (Risinger et al. 1995), and acute i.v. self-administration of nicotine is achieved with 0.03 mg kg^{-1} per injection, but not with 0.02 or 0.04 mg kg^{-1} per injection (Martellotta et al. 1995). In contrast, the effective dose range is wider for anxiolytic effects measured by entries into the open arm of an elevated plus maze (0.1–1.0 mg kg^{-1}) (Brioni et al. 1993).

Summary

In many regards, the nicotine-elicited responses of mice are comparable to those seen in rats, such as the inverted U-shaped dose–response relationship for the complex behavior of nicotine self-administration (see "Intravenous nicotine self-administration"). However, mice are not just small rats. Mice are less sensitive to the effects of nicotine, their metabolism is much faster ($t_{1/2}$= 6–7 min compared to 45 min in the rat), and some strains are more susceptible to the stress of handling and injection. Investigators are strongly encouraged to characterize their own dose–response relationships based on the test or behavior under investigation and the strain of mouse involved. Mice provide a multiplicity of genetically defined inbred strains, as well as specific homologous recombinant deletions or mutations, with which to study the mechanisms underlying the variation in neurobiological responses to nicotine.

In vivo nicotine dose selection in *Drosophila melanogaster*

Introduction

D. melanogaster is one of the most intensively studied organisms in biology and has provided crucial insights into the developmental and cellular processes that are conserved

in mammals, including humans. Flies have a relatively sophisticated nervous system (approximately 300,000 neurons) and are capable of many complex behaviors (DeZazzo and Tully 1995; Hall 1994, 1998; Sokolowski 2001). They are inexpensive and easy to rear in the laboratory and their life cycle is only ~10 days. The major advantage of flies is the simplicity and scale with which they can be manipulated genetically; nearly a century of fundamental genetic analysis has led to the generation of a large number of sophisticated genetic tools. In addition, the past 25 years have witnessed the development of many powerful molecular genetic techniques utilizing flies. Finally, an analysis of the *Drosophila* euchromatin sequence has revealed a high degree of molecular similarity between flies and mammals. For example, *Drosophila* has most, if not all, of the major mammalian neurotransmitters, as well as the molecules involved in synaptic vesicle release and recycling, receptors and channels for neurotransmission, and signal transduction mechanisms involved in neural function in mammals (Littleton and Ganetzky 2000; Lloyd et al. 2000).

Drosophila nicotinic cholinergic system

In contrast to mammals, acetylcholine, rather than glutamate, is believed to be the primary excitatory neurotransmitter in flies. The cholinergic locus of *Drosophila*, encoding choline acetyl transferase (ChAT) and the vesicular ACh transporter (VAChT), is organized in a manner that is similar to vertebrates: VAChT is encoded by sequences contained within the first intron of the ChAT gene (Kitamoto et al. 1998). Finally, acetylcholinesterase (AChE), the enzyme that hydrolyzes acetylcholine, is encoded by a single locus in flies and multiple mutant alleles exist (Restifo and White 1990). Mutations in AChE are lethal, but mosaic animals with brains composed of wild-type and mutant cells do survive with developmental and behavioral defects (Greenspan et al. 1980; Hall et al. 1980) and resistance to insecticides (Fournier et al. 1993; Pralavorio and Fournier 1992). Complete loss-of-function mutations in ChAT are lethal, although several temperature-sensitive alleles that cause paralysis when shifted to the restrictive temperature as adults have been found (Kitamoto et al. 1992). These severe and pleiotropic phenotypes are consistent with a prominent role of ACh in the developing and adult nervous system of *Drosophila*.

Drosophila nAChRs

As is the case for insects in general, *Drosophila* do not use acetylcholine at the neuromuscular junction; therefore, their nAChRs are nervous system specific. Homology-based cloning and genome analysis has identified ten receptors with homology to mammalian nAChRs in *Drosophila* (Gundelfinger 1992; Littleton and Ganetzky 2000). Of these, four are alpha-like, three are beta-like, and the remaining three are more related to each other than to any known alpha or beta subunits. Some *Drosophila* alpha subunits can be functionally reconstituted with vertebrate beta subunits in *Xenopus* oocytes or *Drosophila* S2 cells (Bertrand et al. 1994; Jonas et al. 1994). The patterns of expression of these nAChR homologs have not been studied in detail. However, the expression of several subunits and the binding of alpha-bungarotoxin has been shown to be nervous system specific (Gundelfinger and Hess 1992). Mutations in nAChR subunits have, to our knowledge, not been reported.

Acute nicotine exposure

Delivery by injection

For calculations of the internal nicotine concentrations in injected flies, the volume of an adult fly can be assumed to be ~2 μl, as the internal distribution of nicotine is not known in the injected flies, however, concentrations are necessarily approximations. Nicotine (40 nl in modified physiological saline) has been microinjected into larvae, pupae, and adult flies using glass micropipettes (Zornik et al. 1999). The effect of direct nicotine injections on heart rate, measured using a microscope-based optical assay, is dose and age dependent (Johnson et al. 1997; White et al. 1992). At concentrations of 1 mM and higher, nicotine decreases larval and pupal heart rate, whereas in adult flies, concentrations of 0.1 mM and above increase heart rate. Nicotine at 0.5–4 nmol injected into the abdomen of adult flies exhibits a dose–response relationship for viability, in that 1 nmol has no effect, but 2 and 4 nmol cause 30 and 100% lethality, respectively (Manev et al. 2003). Unlike studies in mammalian species, the stress effects of nicotine injection have not been investigated.

Delivery to the "headless" fly preparation

The application of drugs to the thorax opening of a decapitated fly (Hirsh 1998; Yellman et al. 1998) has been used to score behaviors, such as grooming, spinning, and extended hyperactivity, on a qualitative scale (Ashton et al. 2001). At nicotine concentrations greater than 0.5% nicotine (dissolved in 10 mM sodium phosphate buffer, pH 6), all these behaviors are abolished. Using this assay, fly lines that differ significantly in their peripheral responses to nicotine have been identified (Rothenfluh and Heberlein, unpublished observations).

Delivery by volatilization

Ingeneral,2-to4-day-oldadultmale*Drosophila* are used for these experiments. Nicotine free base solutions are prepared by dissolution in either 70% ethanol or water and volatilized off a nichrome coil (McClung and Hirsh 1998). The flies are exposed to the volatilized nicotine for 45 s in a glass vial and then transferred to a viewing chamber (for direct observation or video tracking) or a cylinder (for analysis of negative geotaxis). It is surprising that the vastly different dose–response relationships have been observed in different laboratories, a discrepancy perhaps attributable to the vehicle in which nicotine is dissolved and the volume of liquid vaporized off the nichrome coil (1 or 2 μl). For example, when 1 μl nicotine dissolved in water is vaporized, the dose at which the *d*rug *e*ffect *s*core (DES) is 50% (DES-50) is approximately 7 μg nicotine (Bainton et al. 2000), whereas vaporization of 2 μl nicotine dissolved in water produces a dose–response curve with a DES-50 of approximately 100 μg (Hou et al. 2004). In contrast, volatilization of 1 μl nicotine dissolved in 70% ethanol and allowed to air-dry (to eliminate the ethanol before delivery to flies) produces a DES-50 of approximately 0.6 μg nicotine (Rothenfluh and Heberlein; Fig. 9). We postulate that such results indicate that, during volatilization of larger volumes of nicotine (particularly nicotine dissolved in water), the drug may be trapped in water droplets that precipitate on the side of the vial and, therefore, is not delivered to the flies. It is not known, however, how much nicotine is "inhaled" by flies exposed to volatilized drug.

The interference of nicotine exposure with most normal fly behaviors can be demonstrated in simple and quantitative behavioral assays. In general, exposure to low doses of volatilized nicotine induces intense grooming (within seconds), followed by increased locomotion, jumping, and uncontrolled hyperactivity (Fig. 8a,b), then a period of hypolocomotion interfering with the flies' ability to perform the robust innate negative geotaxis behavior (Fig. 8c), with complete recovery in approximately 10 min. Higher nicotine doses also induce grooming and hyperactivity, but this phase is followed by a period of seizure-like activity (including leg tremors), which precedes a final hypoactive phase. (Bainton et al. 2000).

The "bang-and-hang assay", a modification of the negative geotaxis assay, allows for the measurement of nicotine's effects over a larger range of doses, as negative geotaxis is affected at lower doses and "hanging" ability is lost with higher doses. After exposure to volatilized nicotine for 45 s, the flies' ability to climb up the walls of a vial provides a "climbing score", and then their ability to hang on to the plug once the vial is inverted provides a "hanging score". The combined DES is the sum of the fraction of flies that climb and those that hang on. Figure 9 shows the combined DESs after exposure to 0.4–1.1 μg nicotine at 20 min (Fig. 9a) and at 1 min (Fig. 9b), the time-point at which flies are most affected by the drug. The flies recover their ability to hang on within 30 min of exposure to a highly incapacitating dose of 3.2 μg of nicotine, while their ability to climb is still impaired 60 min after the drug exposure (Fig. 9c). The dose–response curve of nicotine is fairly steep: the flies are unaffected by exposure to 0.1 μg of nicotine (not shown) and fully incapacitated by 1 μg of it (Fig. 9a). It is surprising that, upon exposure to 125 μg of volatilized nicotine, the flies show approximately 50% lethality; however, the flies that do survive require hours to recover from such a high-dose exposure. Finally, the individual flies exposed to progressively higher nicotine doses (50–400 μg) have exhibited decreased locomotion in the *Drosophila* Activity Monitor, an infrared-beam locomotion detection device commonly used to monitor fly circadian rhythms (Hou et al. 2004).

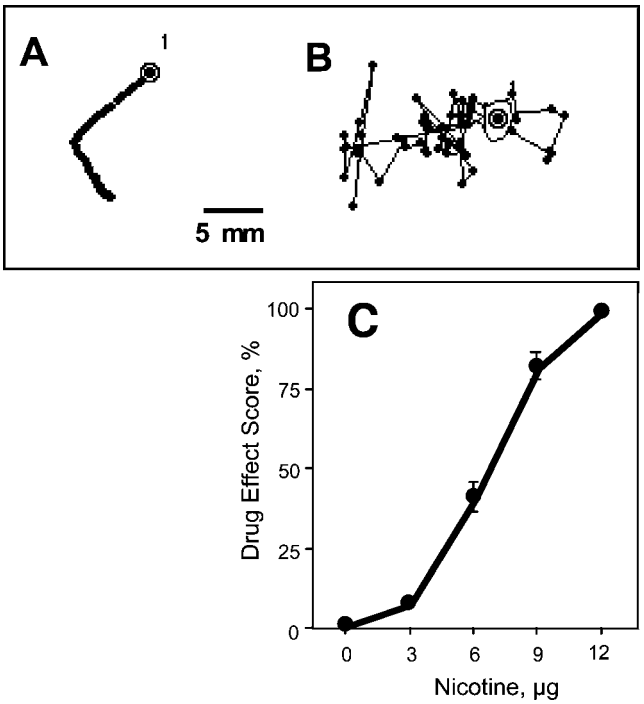

Fig. 8 Simple but quantitative assays can be used to study the interference of nicotine with most normal fly behaviors. Locomotor traces of single flies that have been mock-exposed (**a**) or exposed to 0.5 μg of nicotine volatilized in water (**b**) for a 2-s period; nicotine induces uncontrolled hyperactivity. (**c**) Dose–response curve for volatilized nicotine quantified in the negative geotaxis assay. The drug effect score is the percentage of flies not exhibiting innate negative geotaxis behavior, measured over a 3-min period immediately after nicotine exposure. Drug-treated flies show a dose-dependent reduction in climbing, as well as other abnormal locomotor behaviors (Bainton et al. 2000)

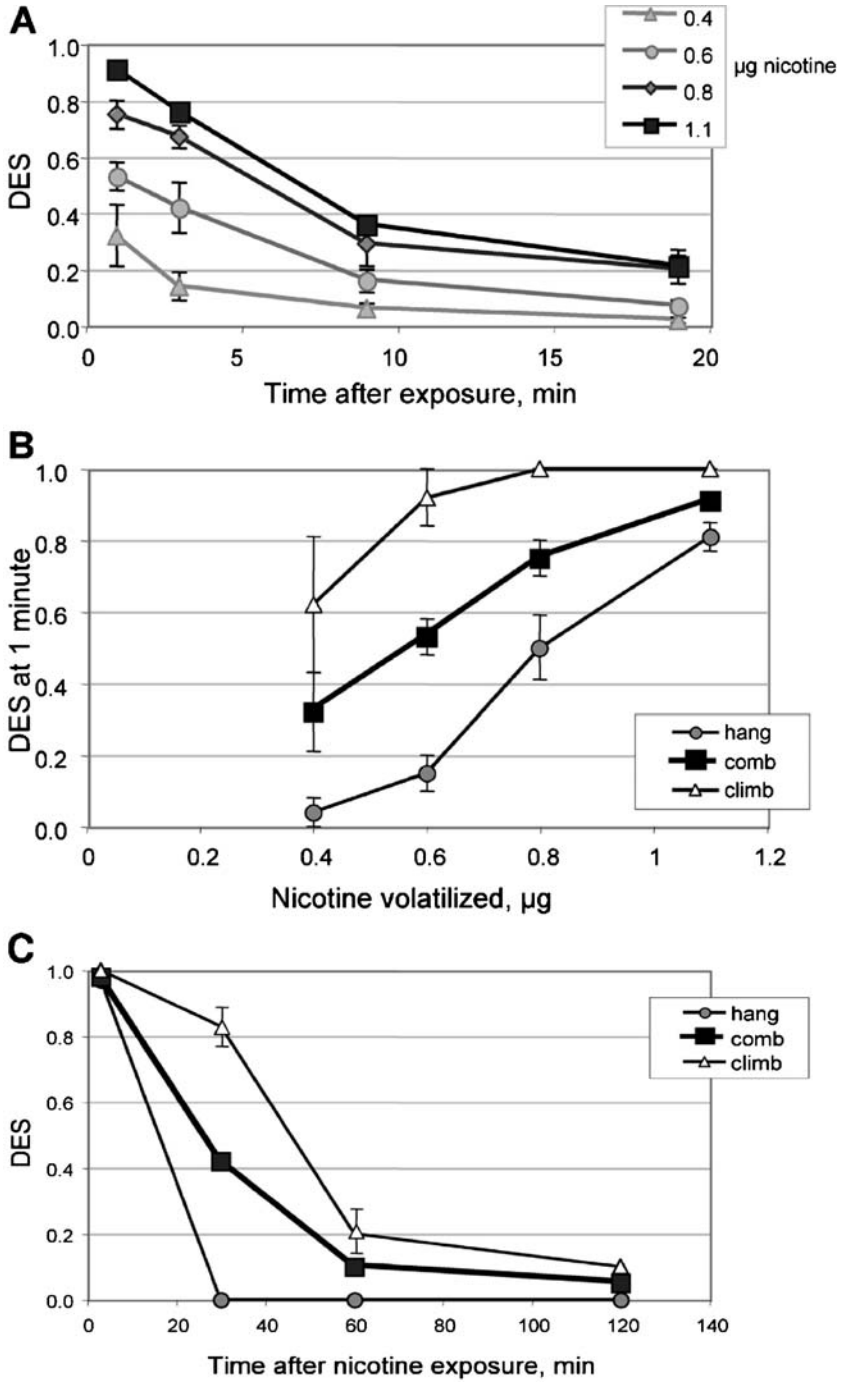

Fig. 9 Behavioral dose–response relationships. Dose–response curves and kinetics of response for volatilized nicotine as measured in the hang-and-bang assay (**a**) can be analyzed separately into "hanging" (*hang*) or "climbing" (*climb*) aspects or as combined (*comb*) score (**b**). The kinetics of recovery can also be analyzed as separate behaviors or as a combined score (**c**) (Rothenfluth and Herberlein, published herein)

Chronic nicotine exposure

Delivery by ingestion

Nicotine in the regular agar- and molasses-based fly food appears to be aversive because, even at non-lethal concentrations of 0.3 mg ml^{-1}, flies do not ingest as much nicotine-laced food as regular food (as measured with the food color FDC Blue #1). Furthermore, even after being starved for a number of hours to increase intake, the flies regurgitate the just-ingested nicotine solution, a behavior not observed with carrier solution (100 mM sucrose) alone

(Rothenfluh and Heberlein, unpublished observations). Nevertheless, chronic nicotine feeding can be used to measure survival rates.

Figure 10 shows the LT_{50} (the time required for 50% of flies to die) as a function of nicotine concentration, with minimal adult lethality at concentrations below 1 mg ml^{-1} (free base) but an LT_{50} of 3.2 mg ml^{-1} nicotine after approximately 36 h. In a similar study, in which survival time was identified as that required for all ten exposed adult flies in a vial to die, 3 mg ml^{-1} nicotine resulted in a mean survival time of approximately 2 days (Carrillo and Gibson 2002). At the highest concentration of 10 mg ml^{-1}, the flies develop seizures upon placement on nicotine food and they die soon thereafter, without even ingesting the food. Such lethality presumably can be attributed to acute nicotine over-exposure through diffusion or sublimation out of the food. The continuous sublimation of nicotine from the food, which presumably happens at all doses, may also explain why nicotine-laced food that is a few days old loses its potency. Therefore, it is recommended that the flies be transferred to freshly prepared nicotine-containing food every 2 days.

Genetics and behavior

Genetic background

Isofemale lines with different genetic backgrounds exhibit different survival times on nicotine-containing food (Carrillo and Gibson 2002). The two wild-type strains, Canton-S and Berlin, also show differences in their behavioral response to volatilized nicotine, in that Berlin flies display a slower recovery of negative geotaxis after nicotine exposure. This may be caused by strain differences in nicotine pharmacokinetics or differences in drive for negative geotaxis (Fig. 11). Therefore, in the context of studying single gene effects, genetic backgrounds must be normalized between experimental and control strains.

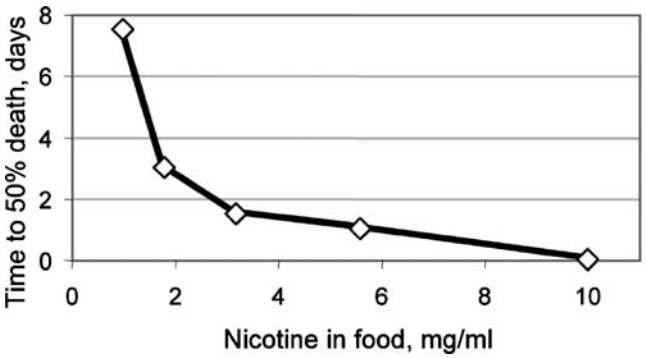

Fig. 10 Effect of nicotine feeding on viability. Flies may find nicotine-laced food aversive, as indicated by a significantly reduced consumption compared to regular food, even after hours of starvation. However, chronic nicotine feeding can be used to measure survival rates, especially at higher doses. The LT_{50} as a function of nicotine concentration is approximately 36 h (Rothenfluth and Herberlein, published herein)

Mutants in the phosphodiesterase gene *dunce* are more sensitive to volatilized nicotine, while mutations in *DCO*, the gene encoding cyclic adenosine monophosphate (cAMP)-dependent protein kinase, result in resistant flies (Hou et al. 2004), implicating the cAMP system in responses to nicotine. Unbiased genetic screens have identified several additional genes that regulate the acute sensitivity to volatilized nicotine (Rothenfluh and Heberlein; Fig. 12). Figure 12 demonstrates the role of a cytochrome-P450-encoding gene in nicotine sensitivity. Hikone R flies are resistant to the lethal effects of nicotine feeding but are only marginally resistant to volatilized nicotine (Fig. 12a,b). Hikone R flies have been shown previously to be resistant to insecticide (DDT)-induced lethality, an effect attributed to overexpression of the P450 CYP6G1 gene (Daborn et al. 2001; Daborn et al. 2002). Two P-element insertions isolated in the genetic screen for mutants with altered behavioral responses to nicotine conversely show strong changes in their response to volatilized nicotine but do not significantly differ in their sensitivity to chronically fed nicotine (Fig. 12c,d). These observations together indicate that the molecular processes regulating the sensitivity to nicotine-induced lethality are genetically separable from those that regulate acute behavioral responses to volatilized nicotine.

Behavior

In the nicotine injection assay, the fly's developmental stage has a profound, qualitative interaction with nicotine on heartbeat frequency: nicotine induces a decrease in heart rate in larvae and pupae but an increase in adults (Zornik et al. 1999). There is a slight trend towards increased resistance to nicotine-induced behavioral effects with age, although the differences are small and have not been systematically investigated. In addition, the gender-dependent differences in the behavioral effects of volatilized nicotine are inconsistent. However, females do show significantly longer survival times on nicotine-containing food (Carrillo and Gibson 2002). It is noteworthy that females are approximately 60% heavier than males and are also more resistant to starvation. Finally, the smaller flies obtained from crowded rearing conditions are more sensitive to nicotine-induced lethality by feeding, compared to flies reared under optimal conditions.

An injection of 4 nmol of nicotine into adult fly abdomens results in complete lethality. However, pretreatment with a sublethal dose (0.5 or 1 nmol 24 h before the 4-nmol challenge dose provides significant protection from

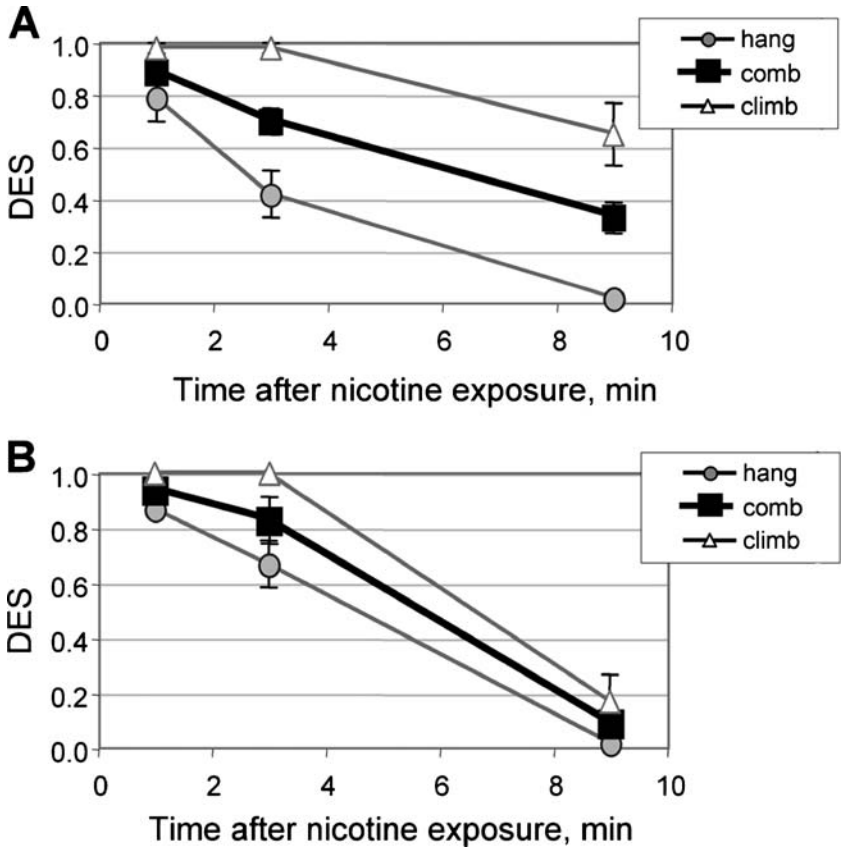

Fig. 11 Effect of genetic background on sensitivity to volatilized nicotine. The effect on "hanging" (*hang*) and "climbing" (*climb*) as well as the combined score (*comb*) are shown for wild-type Berlin (**a**) and Canton S (**b**) flies (Rothenfluth and Herberlein, published herein)

lethality, perhaps through the development of tolerance (Manev et al. 2003). In contrast, repeated exposure to volatilized nicotine results in sensitization, and a second dose delivered 4 h after the initial exposure elicits a stronger drug effect (Hou et al. 2004). These data indicate that, although prior nicotine exposure can alter subsequent responses, a specific effect is dependent on dose and/or route of administration.

Summary

Drosophila possess a relatively sophisticated nervous system, well-characterized complex behaviors, and developmental and neurobiological processes that are conserved in mammals. *Drosophila* have most, if not all, of the major mammalian neurotransmitters, as well as the molecules involved in many signal transduction mechanisms underlying neural function in mammals. Fly nAChRs are nervous system specific, and ten receptors with homology to mammalian nAChR subunits have been identified by homology-based cloning and genome analysis However, pharmacology cannot be predicted by sequence homology, making direct comparisons unfeasible until fly-specific nicotine receptor pharmacology has been characterized. In additon, nicotine metabolism and clearance in *Drosophila* have not received much attention to date. Nicotine dose–response relationships for genetic background and behavior have been demonstrated, e.g., molecular processes regulating sensitivity to nicotine-induced lethality are genetically separable from those regulating acute behavioral responses to volatilized nicotine. Given the sophistication of *Drosophila* genetic analyses as well as the simplicity with which flies can be manipulated with high throughput, findings on the interaction of nicotine in genetic, molecular, and behavioral factors will provide insight for subsequent experiments in higher species.

In vivo nicotine dose selection in *C. elegans*

Introduction

C. elegans is an outstanding model system in which to address nicotinic signaling at physiological, genetic, and behavioral levels (Schafer 2002). The position, lineage, and connectivity of all 302 neurons are well characterized. Electrophysiological studies are possible using extracellular (Raizen and Avery 1994) and patch-clamp recording techniques (Richmond and Jorgensen 1999), as well as in

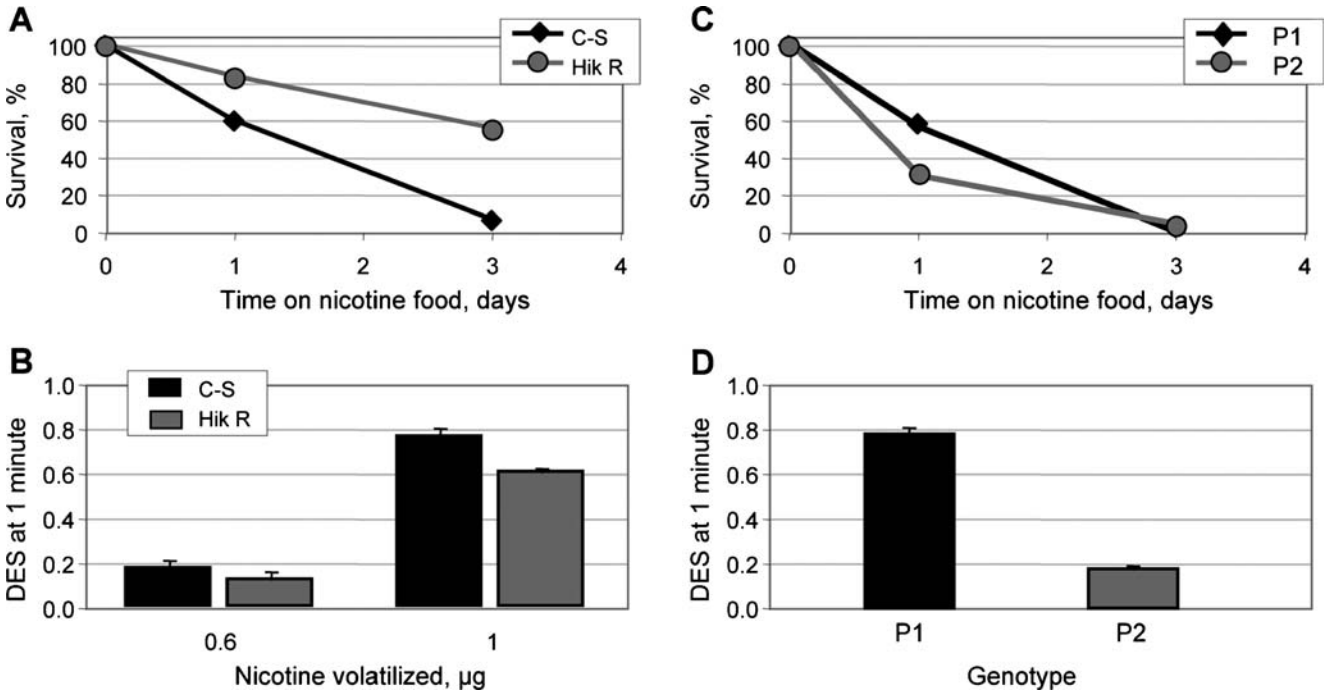

Fig. 12 Genetic dissociation of nicotine-induced lethality and acute responsiveness to volatilized nicotine. (**a**) Hikone R (*Hik R*) flies are resistant to the lethality caused by ingestion of nicotine-containing food, compared to Canton S (*C-S*) flies, but show comparable responsiveness when exposed to volatilized nicotine (**b**). In contrast, although strains P1 and P2 (corresponding to P-element-induced mutations) show comparable sensitivity to the effects of ingested nicotine on viability (**c**); sensitivities to the acute effects of volatilized nicotine are significantly different (**d**). The DES measured 1 min after nicotine exposure is shown. Such findings indicate that the molecular processes underlying these behavioral responses are genetically separable

vivo optical imaging of calcium transients using genetically encoded calcium indicators (chameleon) (Kerr et al. 2000). *C. elegans* is highly genetically tractable; in addition to existing classical mutants and those being generated by the *C. elegans* knock-out consortium, it is also possible to reduce gene function using double-stranded RNA interference (RNAi) (Kamath et al. 2003). The overexpression of tagged molecules allows for visualization of changes in gene expression levels and subcellular localization of proteins in response to nicotine (Gottschalk and Schafer 2006; Waggoner et al. 2000). Nicotine affects many behaviors in worms, including the rate of pharyngeal pumping, body wall muscle paralysis, egg laying in hermaphrodites, and spicule ejection in males.

Uptake and metabolism

Drug uptake is a critical issue because the cuticular exoskeleton of *C. elegans* presents a significant barrier to virtually any drug targeting the neuromusculature. Though a few comprehensive studies have been conducted, it is generally assumed that drug concentrations in body fluids are several orders of magnitude lower than their exogenous concentration in the growth medium. Several studies using nicotinic agonists and antagonists support this assumption. For example, in a comparison of the sensitivities of intact and dissected animals to body muscle hypercontraction induced by various nicotinic agents, exogenous nicotine caused spastic paralysis of intact animals at a concentration of 10 mM, whereas dissected “cut worms” were paralyzed at a concentration of 0.1 mM (Lewis et al. 1980a). A nicotine-sensitive receptor conductance activated by nicotine concentrations as low as 1 mM has also been identified electrophysiologically (Richmond and Jorgensen 1999). Therefore, it is critical that a nicotine dose–response curve be generated for any new behavioral assay in intact animals.

The $t_{1/2}$ of nicotine in *C. elegans* is unknown. In most experiments described below, nicotine is in constant exogenous supply throughout the exposure period. However, the length of time required to clear nicotine from the body once drug is removed has not been investigated nor have the pathways required for nicotine metabolism been described. The *C. elegans* genome contains at least 60 putative CYP450 genes (Gotoh 1998); however, a clear CYP2A6 homolog or coumarin 7-hydroxylase encoding gene has not been identified.

Cholinergic receptors

The best electrophysiologically and pharmacologically characterized cholinergic synapse in *C. elegans* is at the

neuromuscular junction of the body wall, which contains one inhibitory GABAergic receptor and two excitatory acetylcholine receptors (Richmond and Jorgensen 1999). One of these AChRs is primarily sensitive to the anthelmintic levamisole and requires the UNC-38 and UNC-29 subunits (Fleming et al. 1997; Lewis et al. 1980b). The other receptor is primarily nicotine sensitive and requires the ACR-16 subunit (Francis et al. 2005; Touroutine et al. 2005). Both contribute equally to the activation of the muscle cell (Richmond and Jorgensen 1999). The sequencing of the *C. elegans* genome (C. elegans Sequencing Consortium 1998) has identified over 40 putative nicotinic acetylcholine receptor subunits (Bargmann 1998). Some are homologous to vertebrate α- and non-α subunits and some to other insect nAChR subunits, while others appear unique to nematodes (Jones and Sattelle 2004). However, sequence homology cannot be used to predict pharmacology and little characterization of receptor pharmacology has been done in worms, making comparisons between species difficult.

Acute nicotine exposure

Nicotine is typically administered to intact worms by diffusion through the cuticle. This can be achieved either by adding nicotine (free base) to solid nematode growth medium (NGM) plates or by placing the worms in liquid medium containing nicotine. However, the exposure to a given concentration of nicotine in solid NGM is *not* experimentally comparable to the same concentration of nicotine in liquid medium. This may due either to a difference in total surface area in contact with the drug or to differences in osmoregulation or cuticular permeability under different osmotic conditions in each procedure. The concentration and duration of drug exposure vary with the hypothesis, but short-term effects can be seen in less than 1 h and long-term effects within 24–36 h.

Exposure to relatively high nicotine concentrations of nicotine, ranging from 20 to 30 mM in liquid culture for wild-type (*wt*) young adults, causes rapid paralysis of body wall muscles within 10–15 min. This is followed by a slower recovery period within 45–60 min after exposure, during which worms acquire tolerance. The genetic contributions to these phenomena have been studied using RNAi in the *rrf-3* background (see below). Reduction of mRNA for the nAChR subunit *unc-63* or the cubilin *lev-10* results in an enhancement in the acquisition of tolerance (Cregg, Craig and Schafer, unpublished results).

Chronic nicotine exposure

Chronic nicotine exposure produces adaptation, which can be demonstrated by studies of egg laying behavior. Untreated *wt* worms lay eggs in a predictable temporal pattern, composed of bursts of egg laying (clusters), followed by periods of egg retention (inter-cluster interval) (Table 5). An overnight (16 h) exposure of *wt* worms to 30 mM nicotine in NGM causes a shortening of the egg-laying cluster and a lengthening of the intercluster time interval. This effect is still partially seen 24 h after removal from drug.

One mechanism mediating this process is a decrease in UNC-29 receptors (the *unc* designation refers to genes whose mutation results in an uncoordinated phenotype). In an UNC-29::GFP chimera, chronic nicotine exposure leads to a slow reduction in the abundance of UNC-29 in the vulval muscle cells, requiring 12–24 h for maximal effect (Waggoner et al. 2000). Genetic experiments have shown this process to be TPA-1/PKC dependent (Fig. 13).

Genetics and behavior

Genetics

Studies of nicotinic signaling in *C. elegans* began over 30 years ago with the isolation of mutants resistant to the cholinergic anthelmintic levamisole (Brenner 1974; Lewis et al. 1980a,b). The identification of the first nicotinic acetylcholine receptor subunits followed later (Fleming et al. 1993; Squire et al. 1995; Treinin and Chalfie 1995). Additional genes for receptor processing, maturation, trafficking, and assembly also have been identified. These include: the gene encoding the integral membrane protein *ric-3*, required for receptor maturation (Halevi et al. 2002); *lev-10*, required for AChR clustering (Gally et al. 2004); and the less well-characterized *unc-50* and *unc-74* (Brenner 1974; Lewis et al. 1980a,b). Downstream of receptor binding, identified genes mediating nicotinic signaling include the *unc-68* ryanodine calcium channel (Maryon et al. 1996), the *tpa-1* PKC homolog (Waggoner et al. 2000), as well as *lev-9* and *lev-11* (Lewis et al. 1987).

The genetics of *C. elegans* should prove to be a powerful tool in the identification of novel molecules important for nicotinic signaling. In addition to studies using classical mutants, it is possible to examine the effects of reduction of gene expression in live, intact animals using double-stranded RNA-mediated interference. An RNAi library covering 86% of the genome has been developed, allowing for the introduction of dsRNA via feeding (Kamath et al. 2003). While neurons have generally proven resistant to RNAi, new hypersensitive strains, such as the RNA-directed RNA polymerase mutant *rrf-3*, appear to overcome this problem (Simmer et al. 2002). Such protocols are currently being used for high-throughput screening.

Table 5 Effect of long-term nicotine treatment on the temporal pattern of egg laying

Animal type (number, hours, intervals)	Intracluster time constant ($1/\lambda_1$, s)	Intercluster time constant ($1/p\lambda_2$, s)	P	λ_1 (s^{-1})	λ_2 ($s^{-1} \times 10^{-3}$)
N2 (naive; 8, 46, 237)	18±2	1,240±160	0.545±0.035	0.057±0.008	1.5±0.22
Nicotine-adapted N2 (t=0 h; 6, 33, 50)	5±2	3,840[a]±1,080	0.380±0.082	0.203±0.152	0.7±0.25
Nicotine-adapted N2 (t=24 h; 5, 30, 105)	11±2	2,040[a]±1,080	0.490±0.053	0.095±0.021	1.0±0.22
unc-29(x29) (3, 19, 78)	11±2	1,980[a]±480	0.673±0.050	0.090±0.015	0.75±0.38

[a]The intercluster intervals (>300 s in duration) were significantly longer than those in wild type as determined by the Mann–Whitney rank-sum test (p<0.05; Waggoner et al. 2000)

Behavior

The variations in nicotine-induced behavioral response observed at different developmental stages may reflect changes in cuticle properties affecting permeability, particularly relative to molting occurring between larval stages. It may also reflect age-related changes in the nervous system, such as alterations in the pattern of receptor subunit expression, receptor abundance, or intracellular factors regulating nicotinic signaling. These have not been investigated systematically to date.

Some of the behaviors affected by nicotine are gender specific, including egg laying in hermaphrodites and spicule ejection in males. In hermaphrodites, exposure to low nicotine concentrations (0.2–6 mM) in liquid M9 causes a robust, dose-dependent stimulation of egg laying behavior (Fig. 14). For young adult *wt* worms, the half-maximal concentration is 0.8 mM, whereas mutation of the *unc-29*, *unc-38*, or *lev-1* genes leads to a reduction in this response (and decrement in the half-maximal concentration) but does not abolish egg laying behavior.

In males, nicotine exposure causes contraction of the protractor muscles and inappropriate protraction of the spicules, the male-specific structure that facilitates sperm transfer during mating. In liquid culture, 258 μM nicotine in water causes spicule protraction in 90% of *wt* males (Garcia et al. 2001). In *wt* males on solid nematode growth

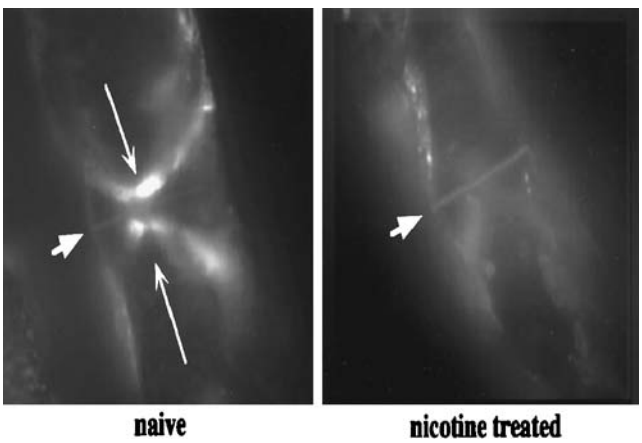

Fig. 13 Effect of nicotine on vulval muscle UNC-29 levels in pmyo-3::unc-29::GFP worms. Vulval muscles (*long arrows*), under control of the muscle myosin promoter *pmyo-3*, in naive and nicotine-adapted ZZ2171 hermaphrodites expressing UNC-29::GFP; *short arrow* indicates the vulva (Waggoner et al. 2000)

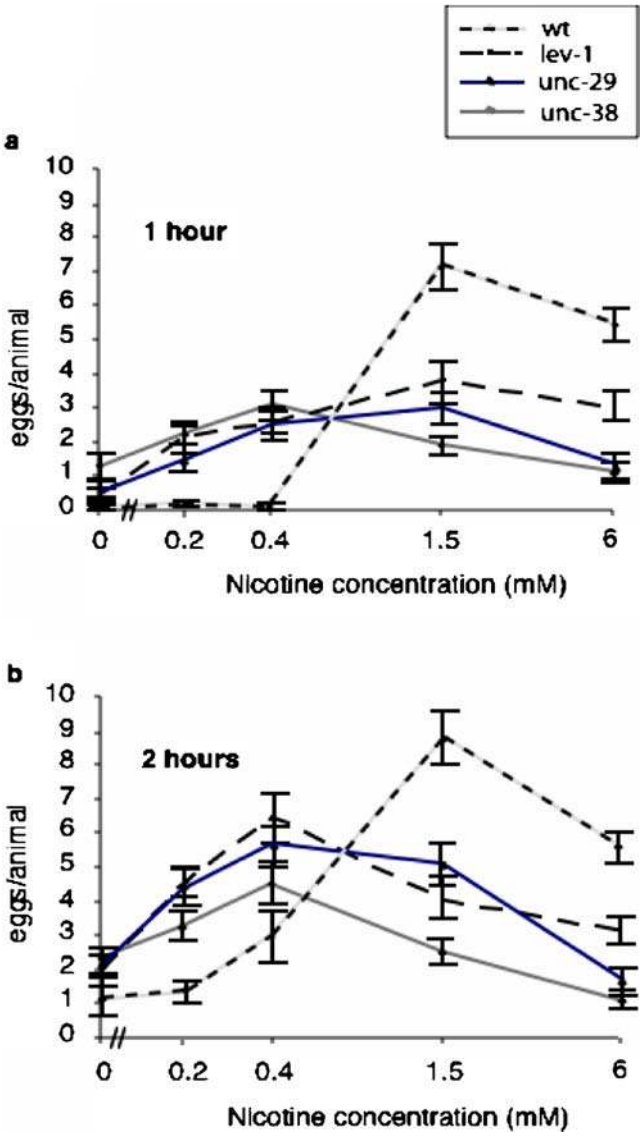

Fig. 14 Effects of levamisole receptor genes on egg laying in response to nicotine. Dose-dependent responses at both 1- (**a**) and 2-h (**b**) post-exposure in wild-type (*wt*) worms are compared to those with *unc-29 (x29)*, *lev-1(e211)*, or *unc-38(x20)* alleles. Data are the mean and standard error of 36 trials at each concentration (Kim et al. 2001)

medium plates, the half-maximal response is also seen at 2 mM nicotine, while the nicotine-sensitive mutant strain *nic-1* show a half-maximal response at <0.1 mM (Fig. 15).

Adaptation to chronic nicotine exposure also occurs in the mating behavior of males. In nicotine-hypersensitive *nic-1* mutants, males exhibit a decreased ability to perform mating after long-term exposure to nicotine. This is likely due to an adaptive failure in the spicule protractor muscles, as well as to those in the body wall and tail, all of which are used in mating behavior (Kim and Schafer, unpublished results). In addition, males are far more insensitive to nicotine-induced body wall muscle paralysis. The basis for this difference is particularly puzzling because males are smaller in body size than hermaphrodites and should presumably be more sensitive, given the difference in surface-area-to-volume ratio. Altered receptor subunit expression patterns or other unknown factors may underlie this discrepancy.

Summary

The well-characterized nervous system of *C. elegans*, its amenability to genetic manipulation, both classic and via chimeras or RNAi, as well as the current focus on receptor subunit identification make this species eminently suitable for high throughput investigations into nicotinic receptor signaling. Relevant age- and gender-dependent behaviors also provide suitable assays for the action of nicotine, as demonstrated by the representative studies in Table 6 that illustrate various behavioral responses elicited by nicotine.

In vivo nicotine dose selection in zebrafish *(Danio rerio)*

Introduction

The wealth of knowledge on zebrafish developmental biology makes it an excellent model system with which to study the effects of nicotine on early embryonic development and the specific nAChR subtypes involved. However, compared to the extensive information published on in vivo nicotine dosaging for the other species discussed elsewhere in this review, the use of zebrafish has only recently entered the field of nicotine research. Therefore, although there is a paucity of current in vivo dosage information, this species is ripe for future studies on the effects of nicotine in zebrafish, especially during development.

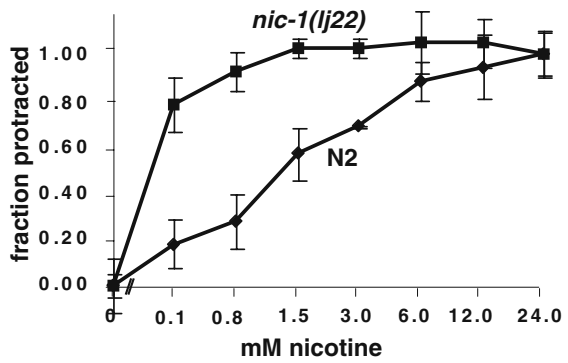

Fig. 15 Nicotine induces male spicule protraction. The half-maximal response is elicited in wt N2 worms at 2 mM nicotine, while the nicotine-sensitive mutant strain *nic-1(lj22)* requires less than 0.1 mM (Kim and Schafer, published herein)

Zebrafish are a freshwater tropical fish available in pet stores and adults are reproductively mature in 3 months (Guo 2004). Zebrafish embryos develop rapidly, with the first somite appearing about 10 h post-fertilization (hpf), compared to 9–10 days in the rat. The embryos develop outside of the mother that has the potential to generate hundreds of embryos from a single mating. Zebrafish embryos are grown in Petri dishes at 28.5°C for several days, allowing for easy observation and manipulation (Westerfield 2000) and making them ideal for studies exploring nicotine effects on development. Early embryos are transparent, providing the ability to observe alterations in specific cells or brain regions throughout development. Neural development occurs in a well-characterized pattern with defined molecular markers available (e.g., antibodies and DNA probes), and brain morphogenesis is quite advanced by 24 hpf (Kimmel 1993; Kimmel et al. 1995; Luo et al. 2001).

Metabolism and clearance

The $t_{1/2}$ of nicotine in zebrafish is unknown and, as nicotine is in constant exogenous supply throughout the exposure period, clearance is also undetermined. Although several zebrafish cytochrome P450 enzymes have been characterized, a zebrafish equivalent of the human CYP2A6 has not been identified. Nicotine exposure can begin immediately after fertilization at the one- to four-cell stage (Kimmel et al. 1995), an advantage not conferred in the rat or mouse developmental models where exposure commonly begins on embryonic day 4 at implantation (Seidler and Slotkin 1990). It should be noted, however, that as the sex of zebrafish embryos cannot be determined, the experiments will contain a mixed population of males and females. Embryos up to 22 hpf are transparent and pigment formation in older embryos can be inhibited by the addition of 0.002% 1-phenyl-2 thiourea to the medium. Embryos up to 5–7 days post fertilization do not require feeding because the yolk cell is still present and nicotine at the desired concentration (usually 5–50 μM) is simply incorporated into the embryo media. The length of exposure and nicotine concentration can be controlled by removal of the embryo

Table 6 Representative studies using nicotine administration in *C. elegans*

Assay or behavior	Nicotine concentration and mode of delivery	Reference
Electrophysiologically measureable activation of receptor in body wall muscle	0.1 mM in saline	Richmond and Jorgensen 1999; Francis et al. 2005; Touroutine et al. 2005
Stimulation of body wall muscle contraction in cut worm	0.1 mM in saline	Lewis et al. 1980a
Tolerance in body wall muscle in intact worm	30 mM in liquid M9	J. Cregg et al., unpublished data
Stimulation of pharyngeal pumping in dissected worm	0.001 mM–0.1 mM in Dent's saline	Raizen et al. 1995
Stimulation of egg laying	0.8 mM in liquid M9	Kim et al. 2001
Adaptation of egg laying	30 mM in solid NGM	Waggoner et al. 2000
Stimulation of spicule protraction	0.258 mM in H_2O_2 mM in solid NGM	Garcia et al. 2001; J. Kim and W. Schafer, unpublished data
Adaptation of spicule protraction	2 mM in solid NGM	J. Kim and W. Schafer, unpublished data

The concentrations are the free base nicotine

media and replacement with fresh embryo media with or without nicotine or other agonists/antagonists. As the stability of nicotine in embryo media is not known, the nicotine-containing embryo media should be changed daily.

Zebrafish cholinergic system and neuronal nAChRs

In adult zebrafish, immunohistochemically identified cells expressing choline acetyltransferase and acetylcholinesterase have been localized. The cholinergic cell distributions in the spinal cord, cranial motor nuclei, olfactory bulb, retina, dorsal telencephalon, tegmentum, and cerebellum are similar to those reported in other vertebrates (Clemente et al. 2004). In addition, acetylcholinesterase-positive cells in developing embryos have been shown to be necessary for normal neuromuscular and neuronal development (Behra et al. 2002; Hanneman and Westerfield 1989). The muscle nAChR alpha subunit gene was cloned after being identified as the defective gene in the paralyzed zebrafish mutant nic1 (Sepich et al. 1998; Westerfield et al. 1990), but no other muscle subunit genes have been cloned to date. Zebrafish motility mutants have been studied (Ono et al. 2001, 2002), but neither the dosages of nicotine required to effect muscle nAChR function nor the affinity of zebrafish muscle receptors for nicotine has been determined.

Three zebrafish neuronal nAChR subunit cDNAs (β3, α7, and α2) have been cloned and display sequence similarity to nAChRs expressed in other species (Zirger et al. 2003; Table 7).

This identification of zebrafish nAChR orthologues also supports the use of zebrafish as a model to study the effects of nicotine acting through specific receptor subtypes. Zebrafish nAChR RNAs are expressed early in development, with the β3 and α2 RNAs detected at 2–5 hpf and the α7 RNA at 8 hpf (Zirger et al. 2003). Two high-affinity [^{3}H]-epibatidine binding sites have been detected in 48-hpf embryos with IC_{50} values of 28.6 pM and 29.7 nM; in 5-day-old zebrafish, the IC_{50} values are 28.4 pM and 8.9 nM, respectively (Fig. 16). Even though specific receptor subtypes have not yet been assigned for each binding site, these IC_{50} values are consistent with the epibatidine binding affinities of neuronal nAChRs in other species (Sharples and Wonnacott 2001). Therefore, although affinities for nicotine itself are undetermined, the agonist epibatidine results indicate that the zebrafish nAChR affinities for cholinergic ligands may be similar to those of nAChRs in other species. This will make it feasible to study the role of specific nAChR subtypes in nicotine's effects using a combination of antisense knockout and nicotine treatment of embryos in culture dishes.

Acute nicotine exposure

Zebrafish have been examined in some behavioral assays similar to those used with other vertebrates (Gerlai 2003) and 5-day-old zebrafish possess locomotor and simple sensory capability, with older zebrafish exhibiting additional behaviors, such as feeding and escape (Guo 2004). Although the first forays into the effects of nicotine on normal zebrafish behavior have been encouraging, a screening for mutations affecting various behavioral responses to nicotine has not yet been reported. A delayed spatial alternation task has been used to study the effects of acute nicotine exposure on memory. Acute exposure (3 min) to low doses of nicotine bitartrate (50–100 mg l^{-1} of water; 16.25–32.5 mg l^{-1} or 38.5–77 μM) improves memory function, while exposure to larger doses impairs memory (Levin and Chen 2004). In addition, the acute effects of alcohol on zebrafish behavior have been examined (Gerlai et al. 2000) and conditioned place preference and dark-adapted visual sensitivity tests have identified cocaine sensitivity in mutant zebrafish

Table 7 Protein sequence identity (%) in pair wise alignments using Geneworks

	Zeb α2	Zeb β3	Zeb α7	Ch α7	Go β3	Mo α2	Go α3	Mo α4	Hu α4	Ch α5	Hu α6	Ch α8	Go β2	Hu β4
Zeb α2		46	37	37	47	70	54	59	57	44	52	37	44	43
Zeb β3	46		34	34	96	48	47	40	40	61	46	33	40	38
Zeb α7	37	34		76	33	35	35	31	32	30	34	63	35	34

The three cloned zebrafish neuronal nAChR subunit cDNAs (β3, α7, and α2) display sequence similarity to nAChRs expressed in other species (Boyd and Zirger, published herein; Zirger et al. 2003). Mouse α2 (Genbank accession #BC011490.1), human α4 (Anand and Lindstrom 1992), mouse α4 (Watanabe et al. 1998), goldfish β3 (Cauley et al. 1990), chicken α7 (Couturier et al. 1990a,b), chicken α8 (Schoepfer et al. 1990), chicken α5 (Couturier et al. 1990a), goldfish β2 (Hieber et al. 1990a), human α6 (Ebihara et al. 2002), human β4 (Tarroni et al. 1992), goldfish α3 (Hieber et al. 1990b)
Zeb Zebrafish, *Ch* chicken, *Mo* mouse, *Hu* human, *Go* goldfish

(Darland and Dowling 2001). Neuronal activity has been imaged in living zebrafish during these various behaviors (Higashijima et al. 2003).

Chronic nicotine exposure

Chronic exposure of zebrafish embryos to 33 μM nicotine (Svoboda et al. 2002), starting at 22 hpf, does not elicit immediate morphological abnormalities, but by 66 hpf embryos are 5% shorter than controls. Embryos at 42 hpf are paralyzed by exposure to 33 μM nicotine, although they still bend in response to tactile stimulation (untreated zebrafish of this age swim vigorously when stimulated). After nicotine exposure between 22 and 66 hpf and then rescue by return to control embryo media, there is a partial recovery of functional behavior between 120 and 168 hpf. Embryos at 120 hpf and which have been exposed to nicotine since 22 hpf remain paralyzed, most likely due to desensitization of muscle nAChRs. The number of spinal secondary motoneurons is dramatically reduced in 66-hpf embryos exposed continuously since 22 hpf. However, if nicotine exposure is limited to 22–66 hpf, there is a partial recovery of functional behavior between 120 and 168 hpf and the number of spinal secondary motoneurons also recovers to near control levels. As such, these transient motoneuron and behavior deficits may be attributable to a delay in the differentiation of embryonic secondary motoneurons and/or altered axonal pathfinding. These motor function deficits are also observed when embryos are exposed to 15 μM nicotine, although no effect is seen at 5 μM (Svoboda et al. 2002). Finally, 2 μM monophosphoryl lipid A (MLA) and 20 μM DHβE block these effects, whereas antagonism by 100 nM MLA is ineffective, indicating that α7 nAChRs may not be involved. In contrast, apoptotic cell death in the zebrafish brain can be produced by exposure to 25–50 μM nicotine bitartrate from 5 to 96 hpf (Boyd et al. 2003). Co-exposure to 20 μM DHβE blocks such nicotine-induced apoptotic cell death, confirming nAChR involvement (Fig. 17). Apart from apoptosis, embryos exposed to nicotine from 5 to 120 hpf do not display any gross morphological abnormalities. In a direct comparison between AB, WIK, and Tubingen strains, 33 μM nicotine elicits comparable behavioral and anatomical alterations, indicating that there currently does not appear to be an underlying genetic susceptibility to nicotine (Svoboda et al. 2002).

Genetics

Several standard wild-type strains are used for most zebrafish research (e.g., AB and WIK strains). Insertional and chemical mutageneses (Amsterdam et al. 2004; Driever et al. 1996) are used to produce a large number of mutant zebrafish, making the search for mutations that modify responses to nicotine feasible. It also is relatively easy to construct strains of transgenic zebrafish expressing new genes or markers, such as green fluorescent protein (GFP), under control of specific promoters (Higashijima et al. 2000; Yoshida and Mishina 2003). The inactivation of specific zebrafish genes has been achieved by injection of antisense morpholino oligonucleotides into the yolk cell of early embryos (Nasevicius and Ekker 2000). A microsatellite genetic linkage map of zebrafish has been completed (Knapik et al. 1998) and sequencing of the zebrafish genome is currently underway at the Sanger Institute (http://www.sanger.ac.uk). Large syntenic regions between human and zebrafish genomes (Barbazuk et al. 2000) will aid in the identification of additional zebrafish nAChRs as well as validate the zebrafish as a model system to explore the effects of nicotine relevant to human nAChRs.

Summary

These early studies using nicotine concentrations ranging from 15 to 50 μM in the embryo media demonstrate the potential of using zebrafish as model for nicotine studies. The ongoing characterization of zebrafish nAChR subunits, coupled with well-identified developmental stages, makes this a promising species. Investigators of nicotine's effects on zebrafish development, memory and other behaviors, are strongly encouraged to incorporate dose–response curves

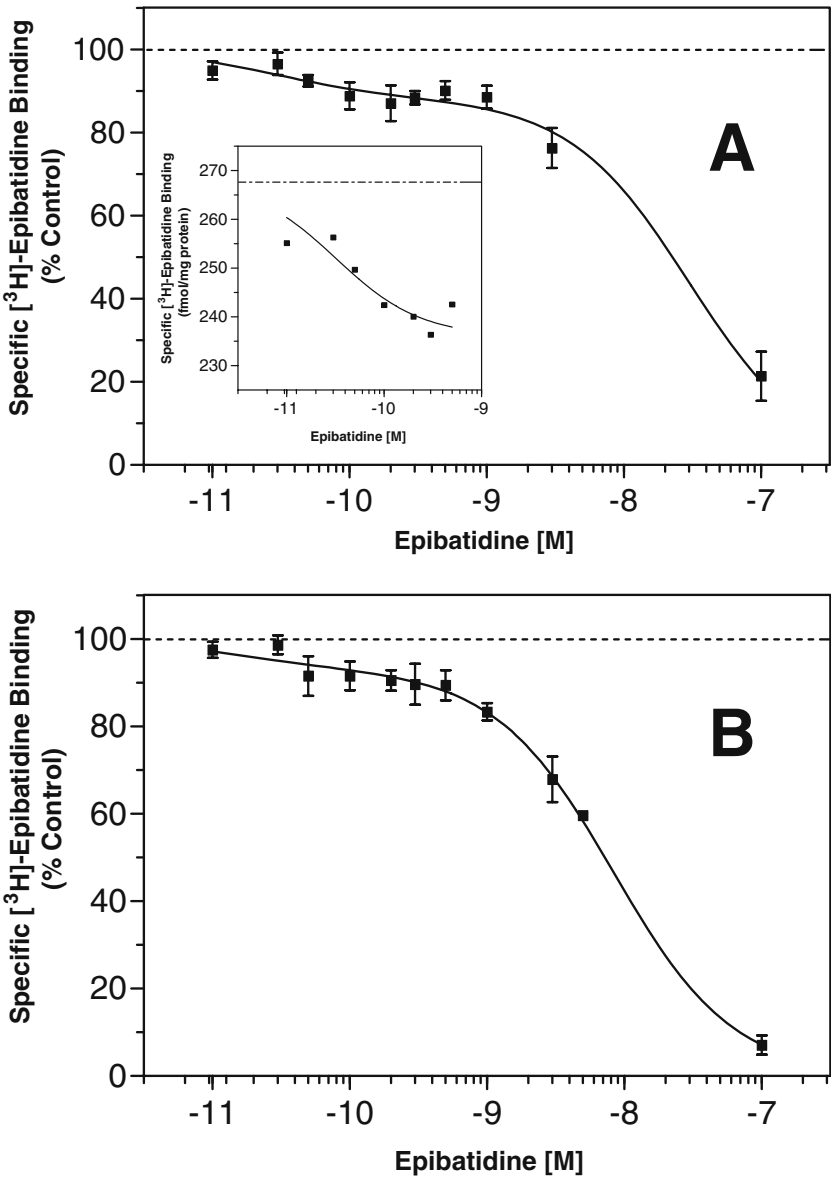

Fig. 16 Homologous epibatidine competition binding studies on membrane preparations from zebrafish embryos, 5 and 2 days post-fertilization (dpf). **a** Epibatidine competition binding (1×10^{-11} to 1×10^{-7} M) in 5 dpf embryos. The data were fit using two-site competition analysis; *dotted* lines are control-specific [^{3}H]-epibatidine binding. One-site analysis of epibatidine competition binding (1×10^{-11} to 5×10^{-10} M; *inset*). **b** Zebrafish embryos at 2 dpf. Data were fit using a two-site analysis; specific binding was defined using 300 μM nicotine. Values represent mean±SEM (n=4 experiments) (Zirger et al. 2003)

for each specific experimental paradigm because of the lack of information on nicotine metabolism and clearance. Well-characterized dosing regimes for specific behavioral and molecular studies will provide the groundwork for a better understanding of zebrafish nAChR function and regional localization.

Conclusions

The rapid rise of nicotine in the blood and resultant high nicotine concentrations in the brain are considered important factors in the reinforcing strength of the cigarette as a nicotine delivery system (Benowitz 1996, 1999). The difference in rate and frequency of nicotine delivery may also have significant effects on the regulation of critical proteins, such as nAChRs and CYP enzymes. As such, in using animal models to study nicotine reinforcement in humans, consideration should be given to the importance of rapid intermittent dosing of nicotine to simulate cigarette puffing. Also note that, in animal studies providing a daily dose of nicotine at a lower dosing rate, such as via osmotic minipumps, blood levels will more closely resemble the trough levels of

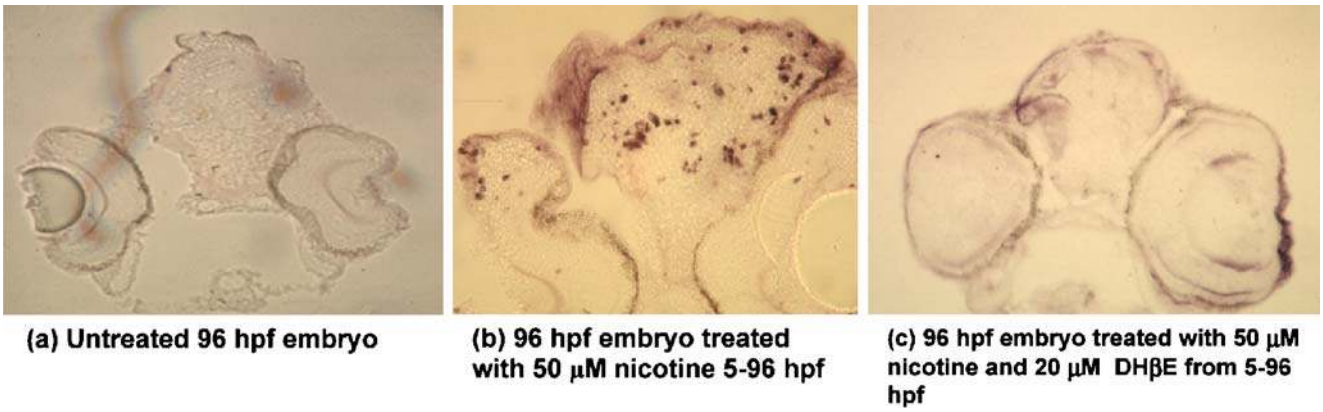

Fig. 17 Nicotine-induced apoptosis in developing zebrafish embryos. Embryos obtained from overnight crosses of adult AB* strain zebrafish were co-exposed to 20 μM DHβE and 50 μM nicotine hydrogen tartrate. The in situ terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling technique was adapted to detect the incidence of apoptotic cells in the developing embryos 96 hpf. The apoptotic cells were visualized with 4-nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate. **a** Coronal section of an untreated 96 hpf embryo; **b** coronal section of embryo treated with nicotine alone from 5 to 96 hpf; **c** coronal section of 96 hpf embryo co-exposed to DHβE and nicotine. Dorsal is toward the *top*; the eyes are visible laterally (Boyd and Zorger, published herein)

nicotine that occur between cigarettes or with NRTs in humans. Nevertheless, in preclinical studies focusing on underlying physiologic and behavioral mechanisms, an empirical determination of the optimal dosages to elicit species-specific responses is essential.

A number of factors may obviate direct cross-species comparisons for many measurements. For example, the levels of nicotine metabolites with potential contribution to the pharmacological profile of nicotine, such as cotinine and nornicotine, may differ between species. The species specificity of the dominant CYP enzyme involved is one determinant, with the mouse isoform more closely approximating human and monkey than rat. A clear CYP2A6 homolog has also not been identified in *Drosophila*. Not only are the *C. elegans* and zebrafish CYPs presently unidentified but the current paucity of information on most metabolic parameters in these species, as well as in *Drosophila*, also precludes cross-species comparisons. In addition, the composition of even nonhuman primate nAChRs has not received the same focus as human and rodent receptors. Finally, despite the increasing number of reports on nAChR subunit sequence homology in non-mammalian models, detailed pharmacological comparisons have not yet been conducted to verify cross-species receptor similarity. The good news is that these species-specific issues have become the focus of a number of laboratories and we should have more answers in the near future.

The authors strongly emphasize that the dosage ranges presented herein are based on specific hypotheses being tested and each is influenced by a substantial number of variables. Issues to consider are route of administration, schedule of dosaging, duration of exposure, underlying physiological status of the subject, and environmental stimuli and drug-associated cues. In vivo dosage regimens used to provide a mechanistic framework for selected behavioral, neurochemical, or receptor-related hypotheses should be individually titrated to elicit optimal outcomes. In contrast, hypotheses related to human consumption via cigarette smoking, NRTs, or in utero exposure should take physiologically relevant plasma nicotine levels into consideration. As for many drugs, the additional complexity with in vivo nicotine dosaging arises from the typical inverted U-shaped dose–effect functions. Therefore, an extrapolation of published doses to any novel investigation requires careful identification of dose–response relationships specific for the new hypothesis being tested and the species used.

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