Invertebrate Models in Addiction Research

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Abstract

While drug addiction is a uniquely human problem, most research examining the biological mechanisms of the transition from substance use to addiction is conducted with vertebrate animal models. Many other fields of neuroscience have greatly benefitted from contributions from simple and manipulable invertebrate model systems. However, the potential of invertebrate research has yet to be fully capitalised on in the field of addiction neuroscience. This may be because of the complexity of addiction and the clinical imperative of addiction research. We argue that the homocentric diagnostic criteria of addiction are no more a hindrance to the use of invertebrate models than they are to vertebrate models. We highlight the strengths of the diversity of different invertebrate model systems in terms of neuroanatomy and molecular machinery, and stress that working with a range of different models will aid in understanding addiction and not be a disadvantage. Finally, we discuss the specific advantages of utilising invertebrate animals for addiction research and highlight key areas in which invertebrates are suited for making unique and meaningful contributions to this field.

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Introduction

Invertebrates are a very large and heterogeneous group including animals without even one single neuron to others with hundreds of millions [Young, 1963] and everything in between. Many key discoveries, often leading to the creation of whole new fields, have only been possible due to the tractability of simple invertebrate animals. Examples include the discovery of neural conduction in squid giant axons [Hodgkin and Huxley, 1945], mechanisms underlying circadian rhythms in *Drosophila* [Konopka, 1987] and molecular pathways of learning in *Aplysia* [Kandel, 2007], to name a few. However, when it comes to understanding complex neuropsychiatric issues, such as addiction, invertebrate research has lagged behind [Burne et al., 2011]. Most research on neuropsychiatric topics has been conducted with mammalian model systems, but there has been a slow shift towards realising the potential of invertebrate animals for understanding the biological mechanisms underlying diseases and disorders [Wolf and Heberlein, 2003; Burne et al., 2011; Scholz and Mustard, 2013]. We review the potential of invertebrate animals to make unique and meaningful contributions to addiction neuroscience.

First, we address how addiction is clinically defined, how drug addiction is being studied in vertebrate and invertebrate animal models and what approaches are cur-

Table 1. Similarities and differences in diagnostic criteria between the two most commonly used psychiatric diagnostic manuals

Name	DSM-IV substance dependence	ICD10 dependence syndrome	Differences
<i>Criteria</i>	three or more of the symptoms listed below occurring at any time during a year	three or more of the symptoms listed below occurring together for at least 1 month or repeatedly for shorter periods during a year	
<i>Symptoms</i>			
Physiological symptoms			
Tolerance	√	√	
Withdrawal	√	√	
Psychological symptoms			
Lack of control	√	√	split into two distinct symptoms in the DSM, one focusing on desire and the other on behaviour
Time usage	√	√	split into two distinct symptoms in the DSM, one focusing on time spent and the other on giving up other activities
Continued use despite harm	√	√	knowledge of harm is not necessary in the ICD
Compulsion	X	√	the DSM does not consider compulsion a symptom
√ refers to criteria present in the diagnostic manual. X refers to criteria that are absent.			

rently available to researchers using invertebrate animals. Then we discuss the core issues of whether there is any evidence that drugs of abuse are rewarding to invertebrates, and whether they have been shown to persist in drug seeking/using despite known harmful consequences. We consider, in detail, the potential challenges of using animal model systems with markedly different anatomies and potentially different targets for some of the drugs of abuse than humans. Finally, we highlight the benefits of working with invertebrates in terms of how they can add to our understanding of addiction.

Studying Addiction with Animal Models

Drug addiction is clinically referred to as substance dependence by the American Psychiatric Association in their Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and dependence syndrome by the World Health Organization in their International Classification of Diseases (ICD10). Like all neuropsychiatric disorders, current clinical definitions of drug addiction are formulated by diagnostic criteria applicable only to humans [Nestler and Hyman, 2010; Fernando and Robbins, 2011]. Only two of the symptoms among the diagnostic criteria

are physiological in nature (tolerance and withdrawal), while the rest are psychological (table 1). These psychological symptoms are subjective and comprise a perceived lack of control, excessive time and resources invested in procuring, using or recovering from drugs and continued drug use despite harmful consequences or compulsion [World Health Organization, 1993; American Psychiatric Association, 1994]. According to current diagnostic criteria, it is not possible to make a diagnosis of addiction based on the physiological symptoms alone (table 1). Therefore, contention remains whether any animal can ever be said to be addicted in the same way as when the term is applied to human addicts [Lester and Freed, 1973] (but see: [Deroche-Gamonet et al., 2004]).

This challenge has, however, not limited work with vertebrate models. Indeed, most approaches aimed at understanding the biological mechanisms underlying the development of addiction have been conducted with rodent models [Chao and Nestler, 2004]. We argue that the human-specific definition of addiction need not limit meaningful invertebrate research. Animal models can be used to study objectively measureable traits that are elements of addiction or drug use such as physiological tolerance or withdrawal or drug taking despite harmful consequences [Sanchis-Segura and Spanagel, 2006]. It is pos-

Table 2. Behavioural traits studying drug exposure in invertebrate animals

	Reward	Neuroadaptations	Learning and memory	Intoxication	Other
<i>Depressants</i>					
Alcohol	self-administration: bee [1–3], fly [4–6] despite harm: fly [4, 5] preference: bee [7], fly [4–6, 8] CPP: crayfish [37–39]	tolerance: fly [9–15], nematode [16] withdrawal: fly [4], nematode [17]	bee [18] fly [19]	bee [20–22] crayfish [23] fly [14, 24–29] planarian [30]	dependence: fly [19] relapse: fly [4, 5] self-medication: fly [31] aggression: bee [32, 33] decision making: bee [34] social behaviours: bee [35, 36] analgesia: crab [40]
Morphine		sensitisation: crayfish [39]			
<i>Stimulants</i>					
Amphetamine	CPP: crayfish [41]; planarian [42]	withdrawal: planarian [43]			exploration: crayfish [44]
Caffeine	preference: bees [45]		bee [46, 47]		wakefulness: fly [48]
Cocaine	CPP: crayfish [41, 49]; planarian [50] altered reward perception: bee [51]	tolerance: bee [52] withdrawal: bee [51], planarian [43, 53–55] sensitisation: crayfish [56], fly [57–61], planarian [62] withdrawal: planarian [70, 71]	bee [51] snail [63]	fly [64] planarian [65–68]	
Methamphetamine	CPP: planarian [69]			planarian [66]	
Mephedrone				planarian [66, 72]	
Nicotine		withdrawal: planarian [73]		planarian [66, 73, 74]	
<i>Cannabinoids</i>					
WIN 55,212-2		withdrawal: planarian [43, 53, 75]		planarian [76]	

References cited here are supplied in the online supplementary document S1 (see www.karger.com/doi/10.1159/000355506).

sible to study elements that contribute to the addiction state in animals, even if it is arguable whether it is possible to capture the full human-specific addiction syndrome in any animal model. Similar issues exist for all neuropsychiatric disorders, because of the reliance on subjective symptoms and the declarative reporting of these [Nestler and Hyman, 2010]. Therefore, throughout this review, when referring to the study of addiction in animal models, we mean the study of traits associated with the behavioural and physiological expression of human addiction. Having dissected the complex and potentially uniquely human concept of addiction into objectively measurable modules, it is necessary to ask which of these modules can be meaningfully studied in invertebrates.

Physiological traits observed following repeated drug administration in mammalian model systems also occur in invertebrate animals (table 2). Clearly, invertebrate animals are well suited to studying the neuroadaptations that follow repeated drug administrations. However, humans consume drugs of abuse because they are inherently rewarding [Siegel, 2005], not for their ability to induce neuroadaptations. Consequently, it is important to assess whether drugs of abuse are also rewarding to invertebrates.

Drug Reward in Invertebrate Animals

Reinforcement is, without a doubt, the main reason why humans seek out drugs of abuse [Siegel, 2005], and it is the most well-studied aspect of drug use and addiction in rodents [Tzschentke, 2007], but the phenomenon of drug reward presents something of an evolutionary paradox [Sullivan et al., 2008]. Many of the most commonly abused drugs (cocaine, caffeine, nicotine, cannabis, opium and heroin) are plant secondary-defence compounds that are thought to have evolved to deter or kill herbivores or parasites of plants [Nathanson et al., 1993; Rattan, 2010]. We should expect most botanical drugs of abuse to have an evolved function to kill invertebrates and to not be rewarding to them. Therefore, if invertebrate animals are to be used for addiction research, the burden of proof remains on invertebrate researchers to demonstrate that drugs of abuse that are rewarding to humans are also rewarding to invertebrate animals. So far, this has been demonstrated for a range of drugs (table 2).

The two main paradigms used to study drug reward in rodent systems are willingness to self-administer and conditioned place preference (for a detailed description

of how these assays are applied in rodents see: [Sanchis-Segura and Spanagel, 2006]). These have only recently been adapted for invertebrates. The self-administration paradigm allows an animal to actively choose to take drugs and to control the amount of the drug being delivered (e.g. a rat controls the number of drug infusions it receives by pressing a lever); this can be said to be the assay that best mimics how humans would use drugs. This paradigm works well with rodents, as they very readily learn operant conditioning tasks. While many invertebrates can perform operant learning tasks [Brembs, 2003; Perry et al., 2013], the self-administration protocols used with vertebrate animals generally require implanting a cannula and having freely moving (but caged) animals connected to a syringe for hours at a time [Sanchis-Segura and Spanagel, 2006].

Cannulas allow for administering the drugs into circulation or directly into target areas in the brain for brain-region-specific studies. The effects of a drug can be markedly different based on the route of administration because of differing pharmacokinetic effects [Samaha and Robinson, 2005]. It is therefore important that the drug is delivered to the central nervous system in a way that is as similar as possible, with regard to the pharmacokinetics, to the way humans use it. For most drugs of abuse, humans have devised methods to rapidly introduce them to the brain for the maximum 'hit' (injection, inhalation, smoking or insufflation). The use of cannulas in animal models allows for a more direct comparison between the effects of the drug on animals to that seen in humans. Cannulas also allow for very precise experimental control of the schedule and level of drug treatment.

Simple scale issues have made the use of cannulas with small invertebrate animals difficult. Some progress has been made with larger invertebrates. Crayfish have already been implanted with cannulas for the purpose of manual drug administration [Panksepp and Huber, 2004], but this has not yet been combined with operant self-administration.

Drugs that are ingested orally can easily be self-administered without the additional complications of having to implant cannulas. So far, this has been attempted with alcohol in *Drosophila* and honey bees. *Drosophila* develop a preference for alcohol that increases with repeated alcohol consumption, and prefer consuming a liquid fly diet containing alcohol to a liquid diet on its own [Devineni and Heberlein, 2009]. Honey bees, on the other hand, will consume sucrose solutions with low concentrations of ethanol, but only if there are no alternatives [Abramson et al., 2004]. While technically this is self-administration,

at present, it cannot be argued that bees find alcohol rewarding, as they prefer pure sucrose solutions over sucrose solutions containing ethanol. It is perhaps not surprising that there is a difference between *Drosophila* and honey bees in ethanol preference, given their different ecologies. Flies seek out rotting fruit, the presence of which can be detected by ethanol odours, for egg laying [Schneider et al., 2012]. Honey bees may encounter nectars containing alcohol while foraging, but these would be indicative of decomposition. Alcohol reduces bees' ability to forage and communicate efficiently, and returning alcoholic nectar to the colony will damage the hive [Bozic et al., 2006]; therefore, bees are expected to have evolved a strong aversion to alcohol. These experiments highlight the importance of taking species-specific ecologies into account, as some animals might not be suited for studying particular aspects of drug effects with certain drugs.

An alternative bioassay for drug reward is the conditioned place preference paradigm which involves an animal demonstrating a learned preference for an environment that has been associated with drug treatment. Typically, this involves a habitat with two distinct environments (e.g. half is lit and half is dark). During the conditioning phase, an animal is restricted to one half of the habitat at a time. The animal is placed in one half during treatment with the drug in question, and in the other half when receiving a control treatment. In the test phase, the animal is given access to both environments and is free to choose where to spend its time [Tzschentke, 2007]. It is assumed that any difference in preference in a properly balanced experiment will be due to the effects of the drug. This approach is widely used because it makes it possible to study both rewarding and aversive effects of drug administration, and to assess the rewarding effects of drugs at doses that do not lead to self-administration. Often, animals are not under the immediate pharmacological influence of any drugs during the test phase, which means that it is possible to assess the effects of drugs that are rewarding but impair self-administration performance such as some opioids in vertebrates.

Conditioned place preference assays have been used successfully with a range of invertebrates. In a conditioned place preference paradigm, the planarian *Dugesia japonica* has shown a preference for textures associated with methamphetamine administration. The effect was highly dose-dependent, showing the strongest effect at intermediate doses [Kusayama and Watanabe, 2000]. Similarly, conditioned place preference developed following methamphetamine and cocaine administration in crayfish [Panksepp and Huber, 2004]. It could be argued

that the conditioned odour paradigm used with *Drosophila*, where flies have to choose between walking towards a drug-associated or a non-drug-associated odour [Kaun et al., 2011], is somewhat similar to the conditioned place preference paradigm. However, in the experiments using this technique, ethanol was ingested during conditioning, and it is thus not entirely clear if the preference is due to caloric content or to drug reward [Pohl et al., 2012].

There are other behavioural paradigms that are unique to invertebrates, which also hint at drug reward. Barron et al. [2009] demonstrated that cocaine alters how honey bees perceive rewards. Honey bees can communicate the location of food sources encountered while foraging to their nest mates via a highly stereotyped dance (for a detailed description of this process, see: [Seeley, 1995]). The likelihood that a bee will dance and the tempo of the dance increases with the perceived quality of the food source found while foraging [Seeley, 1995]. Bees were more likely to dance after visiting a food source if they had been treated topically with cocaine [Barron et al., 2009]. The tempo of the dances was also increased in cocaine-treated bees. Both these aspects indicate that cocaine-treated bees rated food sources higher than control-treated bees. While this experiment does not demonstrate that cocaine is rewarding to bees, it does show that cocaine affects the perception of natural rewards.

Because drug reward is such a key aspect of why humans use drugs of abuse, it is essential that the drugs studied in invertebrates are also rewarding to these animals.

So far, studies on invertebrate drug reward have only covered a narrow range of invertebrates and an even narrower range of drugs (table 2). This does not need to be. From the studies above, we hope we have made it clear that it is possible to study drug reward in invertebrates, both by using methods developed for mammals as well as by taking advantage of the unique ecologies of certain invertebrates. However, it is important to remember that while drug reward may motivate initial consumption, demonstration of drug reward does not necessarily demonstrate addiction.

Invertebrate Drug Use despite Known Harm

Continued drug use despite known harm, taken by many as the hallmark of problematic substance use, is a key step towards addiction [Hasin et al., 2006]. This is clearly different from either preferring a context previously associated with drug administration or continuing

to self-administer when the alternative is to not self-administer, since in these paradigms, there are no harmful negative consequences of drug use. By contrast, human drug addicts face a plethora of negative consequences resulting from their continued drug abuse: poor health, loss of income, contracting diseases from shared needles and potentially serving time in prison time for criminal activity associated with obtaining drugs [MacCoun et al., 1996]. Despite an awareness of the harmful impact of their drug use, addicts continue drug abuse. To examine this facet of drug addiction, Deroche-Gamonet et al. [2004] trained rats to self-administer cocaine, and then paired continued administration of the drug with an electric shock. Their experiments showed that some rats persisted in self-administering even when faced with a painful shock. To meet the diagnostic criteria of addiction, the DSM-IV stipulates that the sufferer is aware of known harm, whereas the ICD10 makes no such stipulation (table 1). We can argue that the shock does not deter the rat from using the drug, i.e. it continues drug use despite harm, but we cannot say that the rat is aware that it will experience harm and its consequences by persisting in drug-taking.

Assays like this for rats have been adapted for invertebrates. In a landmark study, fruit flies walked over an electrified grid towards an odour that had previously been paired with an ethanol reward [Kaun et al., 2011]. It could be argued that this is equivalent to continued use despite known harm, as the flies were willing to overcome harm and an aversive stimulus in order to obtain an alcohol reward. However, there is one important difference between this experiment and that of Deroche-Gamonet et al. [2004]. The flies only learned to associate the alcohol reward with the odour, but not that an electric shock was associated with the alcohol reward. When they crossed the electric grid, it was the first time harm had been paired with alcohol administration. Kaun et al. [2011] showed that flies were motivated to cross a shock-grid to obtain an alcohol reward, but not that they persisted in seeking and consuming the alcohol reward once they had learned that it was paired with shock. From this experiment, it is not clear if flies would engage in continued drug use despite known harm. It would perhaps be better to label this ‘drug-seeking despite harm’.

An experiment by Devineni and Heberlein [2009] is better able to show continued drug use despite learned harmful consequences. In their experiment, flies had to choose between consuming a liquid fly diet and the same diet containing ethanol and aversive quinine. The flies still developed a preference for the ethanol solution, but

this took 3 days longer than without quinine. In a second experiment, the flies were trained in the same paradigm with a liquid fly diet and an ethanol-containing diet only, but were then tested 6 days later with a quinine-laced ethanol diet. They still preferred the ethanol diet when tested. The flies in both of these experiments had had to repeatedly consume aversive quinine in order to obtain the ethanol reward, and had thus had ample opportunity to learn about the aversive stimulus.

There is one important caveat that needs to be discussed with regard to the *Drosophila* experiments. Because the ethanol was ingested orally, the potential existed for confounding the effects of drug reward with that of caloric value [Pohl et al., 2012]. This is not an unsurpassable hindrance, as several methods already exist for administering drugs to invertebrates without the involvement of the digestive system.

The results of Deroche-Gamonet et al. [2004], Devine and Heberlein [2009] and Kaun et al. [2011] can all be interpreted in an associative learning context. Rather than the animals being aware of harm associated with drug use, it can be said that the incentive salience of the drug reward is increased after repeated usage, and thus the need to obtain the drug weighs more heavily than the desire to avoid punishment [Robinson and Berridge, 2008]. Seen in this light, is it possible to argue that an animal is aware of potential harm? It is a relatively straightforward matter to obtain a meaningful answer from a patient presenting at a clinic, but it is virtually impossible to argue that any animal is 'aware' of harmful consequences like a human is [Lester and Freed, 1973]. We can, however, interpret these experiments, and even human behaviour, as associative learning processes. Assuming nothing more than an associative process, the criteria of continued use despite learned harmful consequences could be applied equally to rodents and invertebrate models. We therefore see no reason to assume that these kinds of experimental approaches address the concept of 'drug use despite known harm' in a more meaningful way if the animal in question is a rat instead of a fly.

As drug use despite known harm is considered such an important part of the addiction phenotype [Hasin et al., 2006], addressing this issue should be among the top priorities for researchers working on invertebrate models of drug addiction. We can hope that the two studies mentioned above are only the beginning of invertebrate experiments examining drug use despite learned harmful consequences.

Comparative Neuroanatomy of Brain Systems in Humans, Vertebrates and Invertebrate Models

In vertebrate animal models, the brain regions and circuitries studied have clearly relatable and homologous human counterparts [Butler and Hodos, 2005], but in the case of invertebrates, the homology is not clear [Farris, 2008a, b]. Invertebrates are a very diverse group, made up of animals from several very different phyla with many different body plans and a range of nervous systems. This is in sharp contrast to mammals where all animals belong to the same class and body plans and brain structures are largely conserved.

When studying the effects of drugs of abuse in non-human primates or other mammalian model systems, the brain areas affected are the same as those targeted in humans [Koob and Volkow, 2010]. As an example, the brain regions involved in escalated drug consumption in humans, e.g. the dorsal striatum, nucleus accumbens and amygdala [Everitt and Robbins, 2005], are conserved across all mammals, and indeed most vertebrates [Butler and Hodos, 2005]. This is true for both very basal structures as well as what are considered to be higher brain regions. These conserved structures share both functional and structural properties, and findings from one species therefore have direct implications for others (but see: [Uylings et al., 2003]).

The structural homology of brain regions between vertebrates and invertebrates is far less clear (excellent diagrams of the brain structures underlying reward processing systems can be found for insects in the study by Perry and Barron [2013] and in the study by Koob and Volkow [2010] for rodents and humans). There is some evidence that the dorsal nerve cord of vertebrates represents an inverted form of the ventral nerve cord found in invertebrates [Arendt and Nübler-Jung, 1994], suggesting a common origin for the central nervous system in bilateral animals [Denes et al., 2007]. Moreover, it has been argued that both lower [Strausfeld and Hirth, 2013] and higher [Tomer et al., 2010] brain regions may have their origin in structures that evolved prior to the split between the deuterostomes and protostomes based on similarities in structure and developmental genetics. Other studies have interpreted these similarities as the result of evolutionary convergence due to similar constraints and selective pressures [Farris, 2008a, b]. Regardless of whether these regions represent conservation or convergent evolution, they have diverged significantly and shown independent specialisation since the separation of deuterostome and protostome lineages. The neurocircuitry and

specific anatomy of vertebrate and invertebrate brains are markedly different [Chiang et al., 2011], and there are currently no regions recognised as direct homologues for any of the brain regions studied in relation to addiction in mammals (e.g. the hippocampus and amygdala) in any invertebrates (but see: [Strausfeld and Hirth, 2013]). It would be very hard to argue that any findings on a neuroanatomical level would directly translate from invertebrates to vertebrates. However, this does not mean that findings from invertebrates cannot provide useful information about the nature of addiction.

Mammalian models are, and might always be, essential for working out the neurocircuitry of human drug responses, but, in order to understand the nature of behavioural reward systems and what it is that makes these uniquely susceptible to being hijacked by psychotropic substances, there is an enormous benefit to studying simpler circuits. Many invertebrate animals have very small and simple nervous systems. The three most commonly used invertebrate model systems, *Caenorhabditis elegans*, *Drosophila* and the honey bee possess central nervous systems consisting of 302 [White et al., 1986], approximately 100,000 [Shimada et al., 2005] and <1,000,000 neurons [Witthöft, 1967], respectively. Methods exist for investigating the function of individual neurons in both *C. elegans* [Mori and Ohshima, 1995; Yanik et al., 2004] and *Drosophila* (for an excellent review of this methodology in an addiction context see: [Kaun et al., 2012]), allowing researchers to determine the role of not only circuits, but even of individual identified neurons. Nothing like this is currently possible with mammalian models, both because of a lack of genetic toolkits and the overwhelming complexity of the mammalian nervous system.

This approach has been immensely fruitful for understanding the basis of learning and memory [Kandel, 2007]. We think it also would be excellent for understanding the nature of drug reward. Invertebrate models have proved exceptionally valuable for studying molecular and electrochemical signal mechanisms [Hodgkin and Huxley, 1945; Kandel, 2007]. While these can be considered basic physiological mechanisms, invertebrate research has also been useful for elucidating the mechanisms of behavioural systems, even if these systems involve different anatomical substrates in vertebrates and invertebrates.

As an informative example, the molecular machinery underlying the circadian rhythms of animals is conserved between vertebrate and invertebrate animals. Despite biological clocks relying on very different structures and circuits in vertebrates and invertebrates, the operations of

the molecular mechanisms have been found to be very similar, and it has been possible to transfer inferences from invertebrates to inform the operation of mammalian clocks. The molecular underpinning of circadian rhythm was first discovered in *Drosophila* [Konopka, 1987], and was later confirmed to be shared with vertebrate animals [Sehgal, 1995]. While the molecular mechanism underlying circadian rhythmicity is highly conserved, the neural substrate is not. In vertebrates, the job of keeping track of time is done by the suprachiasmatic nucleus [Stephan and Zucker, 1972]; this nucleus does not exist in invertebrate animals. In invertebrates, the structures supporting this function vary between different animals [Hut and Beersma, 2011]. Despite the lack of anything homologous to the suprachiasmatic nucleus in invertebrates, this did not prevent invertebrate models from helping to elucidate the mechanisms of biological clocks.

It could be that a biological clock is such a simple phenomenon that its workings can be performed by any substrate. We therefore turn to a slightly more complex task: non-elemental learning. Non-elemental learning refers to the ability to learn patterns or rules that are not consistent with simple associative learning (e.g. negative patterning: where two odours are reinforced on their own but not when presented simultaneously, or vice versa). This was once thought to only be found in vertebrates due to the requirement of certain specific brain regions that are possessed by these animals [O'Reilly and Rudy, 2001]. However, animals lacking these regions have no trouble in performing non-elemental learning tasks [Perry et al., 2013]. Even *Drosophila* is capable of this complex behaviour [Young et al., 2011]. Furthermore, the molecular mechanisms underlying learning and memory in invertebrates are generally conserved in mammals [Kandel, 2007], and it has been possible to study mechanisms of learning in invertebrates that have translated to vertebrates, even if their specific neuroanatomies are radically different.

If the function of the suprachiasmatic nucleus had been discovered prior to the understanding that invertebrates, and even plants and bacteria, are able to keep track of time, it is possible that we may have assumed that a suprachiasmatic nucleus is a prerequisite for a biological clock (until evidence to the contrary became available). This might seem preposterous, but this was the situation for non-elemental learning until only a decade ago, where it was thought to be a feature only producible by certain structures in the mammalian brain [Giurfa, 2003]. These examples demonstrate that invertebrates can display forms of complex behaviour, even if the specific brain structures

supporting these forms in mammals or other vertebrates are not present. To assume that the behavioural traits linked to addiction in humans are exclusively linked to certain brain regions in the mammalian brain might one day seem as preposterous as assuming that particular parts of the mammalian brain are essential for time-keeping or non-elemental learning across the entire animal kingdom.

Molecular Targets of Drugs of Abuse

The brains of vertebrates and invertebrates are very distinct in terms of their anatomies and circuitry, but they both use many of the same signalling molecules.

The classic neurotransmitters glutamate, γ -aminobutyric acid, acetylcholine and the monoamines (dopamine, noradrenaline and serotonin) are utilised by all animal nervous systems, with only minor variations. There are many functional similarities in the roles played by these signalling molecules across animal groups, e.g. the biogenic amine dopamine appears to be involved in all animal reward circuits across all taxonomic groups investigated so far [Barron et al., 2010]. However, the specific molecular machinery involved, i.e. the synthesis enzymes, receptors and re-uptake transporters, can be quite variable between species [Blenau and Baumann, 2001; Mustard et al., 2005; Chen et al., 2006a].

This variation can be illustrated by comparing the target specificity of cocaine in humans and in fruit flies. In humans, cocaine is a lot more effective in blocking dopamine re-uptake transporters than serotonin re-uptake transporters [Han and Gu, 2006]. In *Drosophila*, it is the other way around [Makos et al., 2009; Vickrey et al., 2009; Borue et al., 2010; Makos et al., 2010]. That there is interspecies variation in how effective cocaine is at blocking different transporters is hardly surprising, given the large amount of variation between biogenic amine transporters between different species [Caveney et al., 2006]. What is surprising though, is that, despite these differences, the main behavioural responses to cocaine in both humans and flies appear to be due to interferences with dopamine signalling [Bainton et al., 2000].

One difference in neuromodulator systems between vertebrates and invertebrates relevant to the modes of action of drugs of abuse is that the biogenic amine noradrenaline is not present in a range of invertebrates. Instead, tyramine and octopamine, which are only present in trace amounts in vertebrate nervous systems [Burchett and Hicks, 2006], play an important role in modulating behaviour and physiology [Roeder, 1999, 2005]. Despite

these differences, for most drugs tested on invertebrates, the behavioural responses are quite similar to those of mammals (table 2).

Some primary targets of human drugs of abuse are completely missing in some invertebrates. For example, in mammals, heroin and opium bind to opioid receptors, which induce euphoria and have strong analgesic properties [Waldhoer et al., 2004]. It is presently unclear what role opioids play in invertebrate nervous systems [Harrison et al., 1994]. There are no opioid receptors or neuropeptides found in either *C. elegans* or *Drosophila* [Selami et al., 2010], and it is currently not clear if opioid receptors even exist in this group of animals. In arthropods, morphine has potential analgesic effects [Lozada et al., 1988], and its administration alters behaviour and is potentially rewarding to crayfish [Nathaniel et al., 2009, 2010], but the molecular targets of morphine in these animals remain unknown.

The targets of cannabinoids in invertebrates are also unclear. The only evidence of the action of cannabinoids in invertebrates comes from studies examining the nematocidal and insecticidal properties of cannabis [Badshah et al., 2004; Kayani et al., 2012] and changes in motor output seen following administration of a synthetic cannabinoid in planarians [Buttarelli et al., 2002]. It was believed that there were no cannabinoid receptors in invertebrates, and that they had only originated after the separation of protostomes and deuterostomes [Elphick and Egertová, 2001], as genes orthologous to mammalian cannabinoid-binding-sites have not been found in insects [McPartland et al., 2001]. Receptor-binding and bioinformatic studies have shown that cannabinoid receptors are present in deuterostome invertebrates (e.g. tunicates and sea urchins). For protostomes, there is no bioinformatic evidence for cannabinoid receptors, but binding studies have shown that it is likely that cannabinoid receptors are present in at least some molluscs, cnidarians, velvet worms and crustaceans [McPartland et al., 2006]. The function of cannabinoid signalling in these animals is currently not known, but it appears that it is an ancient trait that has been secondarily lost from the genomes of insects and other invertebrates where it is no longer present. In mammals, endogenous cannabinoids function as important retrograde neurotransmitters regulating neuroplasticity throughout the brain [Katona and Freund, 2012]. Given the importance of cannabinoid signalling for neuronal function in mammals, it is rather puzzling that it has been lost in so many invertebrate groups.

The lack of receptors for opioids and cannabinoids in insects and some other invertebrate groups does not

mean that these compounds do not have any effect on these animals, as evidenced by the nematocidal and insecticidal effects of cannabinoids [Badshah et al., 2004; Kayani et al., 2012]. It does, however, mean that we should be cautious when using certain animals as model systems for studying the effects of these drugs. The mechanisms by which these drugs have an effect could potentially be completely different, and therefore make it less likely that findings would transfer to any mammalian models. A way to circumvent this problem has been attempted in *Drosophila* by the transgenic insertion of human μ -opioid receptors into the fly genome [Sellami et al., 2010]. At present, it is not yet clear how useful this approach may be.

Despite great differences in circuitry between vertebrates and invertebrates as well as the variation in neurotransmitter systems utilised by the brains of all these diverse animals, we have seen that many of the drugs that are rewarding to vertebrates are also rewarding to invertebrates (table 2). In addition, most drugs of abuse, regardless of their immediate molecular target, exert effects upon dopamine neurons [Bainton et al., 2000] which are involved in reward processing in most animals [Barron et al., 2010]. By investigating the effects of drug administration in a wide variety of model systems, we are more likely to understand what it is about behavioural reward systems in general, and dopamine-modulated circuitry specifically, that yields such complex behavioural responses to drugs of abuse.

Benefits of Working with Invertebrates

Invertebrate addiction research has already led to the discovery of mechanisms that have later been verified in rodents. One such example is the involvement of circadian genes in the development of sensitisation to cocaine following exposure. This was first demonstrated in *Drosophila* [Andretic et al., 1999] and then later confirmed in mice [Abarca et al., 2002]. These genes have also subsequently been shown to be implicated in drug reward in mammals [Abarca et al., 2002; Spanagel et al., 2005]. The exhaustive range of readily available mutants makes the discovery of genes related to addiction much more feasible in invertebrate model systems than in rodents. Despite the increasing number of transgenic mice strains available, it is unlikely that the feasibility of making this type of uninformed molecular discovery will approach that of invertebrates anytime soon.

Many high-throughput powerful techniques for studying gene and genome function are only available in invertebrate systems. One example is that of the honey bee to examine whole-genome DNA methylation patterns following acute or repeated drug exposure [Lyko et al., 2010; Flores et al., 2012; Foret et al., 2012; Herb et al., 2012]. Cocaine administration directly affects the expression of DNA methyltransferase 3, causally linked with de novo DNA methylation, in the nucleus accumbens [LaPlant et al., 2010], and drugs that inhibit these transferases decrease cocaine sensitisation [Anier et al., 2010]. The role of DNA methylation in this process is not yet understood, and it is rather problematic to examine these questions in mammalian model systems due to a relatively high level of DNA methylation combined with relatively large genomes. The classic invertebrate model systems, fruit flies and *C. elegans*, are also not useful when it comes to elucidating the role of DNA methylation in drug addiction (as neither of these organisms have functional DNA methylation systems [Lyko and Maleszka, 2011]). However, other invertebrate animals, such as the honey bee, appear to be excellent model species for understanding the role of DNA methylation. Whole-genome methylation studies are a lot more feasible on bees than on rodent models, for two simple reasons. The genomes of these insects are much smaller than mammalian genomes [Adams, 2000; Weinstock et al., 2006], making the sequencing effort required, for now, quicker and cheaper. In addition, the overall level of DNA methylation in honey bees is much lower than that of rodents (approx. 1 vs. 70% of CpG-dinucleotides are methylated), making it easier to detect small changes in genome-wide methylation patterns [Lyko and Maleszka, 2011]. By examining whole-genome methylation patterns in honey bee brains following single drug exposures, or after the development of specific neuroadaptations, it is possible to create lists of genes likely to be targeted by different drugs or in different contexts, and to then examine these targeted genes for hyper- or hypomethylation in key areas of mammalian brains following similar exposures. Using this approach, we can build up an understanding of the role of DNA methylation in addiction.

Another example is the *Drosophila* genetic reference panel, consisting of 192 fully sequenced *D. melanogaster* strains [Mackay et al., 2012]. This panel allows for drug-associated quantitative traits to be analysed with single-nucleotide resolution. The usefulness of this approach has been demonstrated in relation to ethanol consumption, when a sub-set of 40 selected lines was used to predict a single-nucleotide polymorphism linked to varia-

tions in human alcohol drinking behaviour [Morozova et al., 2009]. The potential of this technique for future genome-wide analysis of components that influence the sensitivity and adaptation to drugs of abuse looks very promising.

Moreover, there are many other fundamental advantages of working with invertebrate models. A single fruit fly can lay over 2,500 eggs during its first 30 days of adult life [Shapiro, 1931], and already 2 weeks after these eggs hatch, there will be more sexually mature adult flies. Combined with virtually non-existent husbandry costs, the number of animals that can be made available for study in a relatively short time is many-fold higher than anything imaginable with mammals. This high output, paired with short life-cycles, renders most invertebrates ideal candidates for examining the effects of repeated drug administration on lifespan, across generations or across multiple lines. These traits are the same that make many invertebrates ideal for large-scale artificial selection experiments. Selection experiments can be tremendously useful for examining both natural variability in susceptibility to drug addiction and how this variability might be changed by a selective regime. It is entirely feasible to sequence multiple lines of *C. elegans* or *Drosophila* before and after a selective sweep to alter behavioural responses to drugs of abuse. Such a study would yield (with single-nucleotide resolution) the genomic loci underlying susceptibility to a drug of abuse. While artificial selection experiments with rodents have provided some very interesting findings [Crabbe, 2002], they are often severely limited by low population sizes and the sheer amount of time it takes to raise multiple generations of rodents. Neither of these concerns exists with the common invertebrate model organisms. It is even possible to fully automate this process in *C. elegans* [Chronis, 2010], to freeze individuals that will return to life when thawed, and thus preserve all genetic changes as they occur during the selection process. This makes it possible to establish multiple independent replicates of several artificially selected lines with the entire selection history available in the freezer.

Studies spanning the diversity of invertebrate nervous systems can explore the basic functional properties of reward circuits, and how drugs of abuse alter their function. In some invertebrate animals, it is possible to study the role of identifiable clusters of neurons and even of individual neurons [Yanik et al., 2004]. This is in sharp contrast to what is possible in rodents, where most studies compare different brain regions or sections of a brain region. This level of resolution could potentially help uncover what it is about animal reward systems that make

them susceptible to being hijacked by drugs of abuse. One of the best-described reward circuits is that of *D. melanogaster* [Waddell, 2013], and the method for investigating how the elements of this circuit are affected by drugs is highly developed [Kaun et al., 2012]. It is currently possible to silence or activate neurotransmission as well as suppress the expression of a single gene in identifiable neurons. While this approach will not describe how human specific circuits are affected, it will tell us something about how drugs affect reward pathways to corrupt reward-seeking behaviour or reward responsiveness at a resolution that is currently unimaginable in any mammalian system. Perhaps the nature of circuits that process and encode rewards have unique vulnerabilities due to their function that can only be uncovered by analysing the role played by individual neurons in these circuits? As finer levels of analyses become available for investigating the function of individual neurons within mammalian brains, we need to be able to make useful predictions about the properties of reward circuits for which we should be looking. Some of this might come from computational neuroscience, but it is very likely that the findings from decades of analysing behaviour at the single-neuron level in invertebrates will be immensely helpful in suggesting functional properties of vertebrate behavioural systems. It is therefore essential that we also start compiling information at this level in the context of addiction research as soon as possible.

Lastly, some invertebrate animals have unique lifestyles and ecologies that make them potentially very interesting for understanding the effects of certain drugs. An example is the cocaine tussock moth, *Eloria noyesi*, whose larvae are obligate feeders of the cocaine plant [Blum et al., 1981]. These moths are able to thrive on the otherwise lethal plants because their dopamine re-uptake transporters are insensitive to cocaine [Chen et al., 2006b]. By studying re-uptake transporters that are insensitive or have a reduced sensitivity to cocaine, we can better understand what makes mammalian re-uptake transporters so sensitive to this drug [Sandhu et al., 2002]. As this example shows, some insights can only be gained by examining the different aspects of drug use and addiction in many independent species.

Concluding Remarks

There is nothing about invertebrates that intrinsically makes them unsuitable for addiction research. When approaching the subject in the same way as addiction/drug-

associated traits are studied in rodents, it becomes clear that the traits can be equally valid in vertebrate and invertebrate model systems, and we have seen that a variety of addiction/drug-associated traits have been successfully studied in invertebrates, including drug reward and continued use despite harm. Being able to study these concepts is not enough. In order for invertebrate addiction research to remain relevant, we need to take advantage of the sophisticated tools available to generate novel insights that cannot be discovered by methods currently available in mammalian model systems. A range of different approaches with a variety of organisms enables us to generate new and radical hypotheses and build a better understanding of why reward systems are vulnerable to drugs of abuse and how addiction develops. However, even if

the concept, behavioural response and many of the molecules involved are the same, it is important to remember that the underlying neurocircuitry is different and the mechanisms involved are not necessarily identical. For this reason, vertebrate and invertebrate research are both needed to fully understand addiction in humans.

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