

Škola doktorských studií v biologických vědách

Jihočeská univerzita v Českých Budějovicích

Přírodovědecká fakulta

# **Mozkový systém odměny u hmyzu**

Disertační práce

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České Budějovice 2023

Tato práce by měla být citována jako:

Dvořáček, J., 2023: Mozkový systém odměny u hmyzu. (Brain reward system in insects, Ph.D. Thesis, in Czech). Jihočeská Univerzita, Přírodovědecká fakulta, Škola doktorských studií v biologických vědách, České Budějovice, Česká republika, 71 str.

### **Anotace:**

Animal behavior is not random; rather, it is primarily determined by the biological significance of environmental stimuli: stimuli essential for an individual or species' survival are marked by specific brain areas, the so-called reward system, with a positive hedonic value and their achievement is associated with the pleasure (reward). The concept of the reward system emerged from research of the mammalian brain; early theories held that it was a system present only in the brains of higher animals (the mesolimbic dopaminergic system), and that reward was a manifestation of more complex neural networks and higher brain functions (as a component of emotional circuits). However, there has been a change in how the reward system study's findings are interpreted. The brain reward has evolved from a mostly solitary phenomenon to a complicated function that is divided into the components of "liking", "wanting", and "learning", and from the predominate role of dopamine to a more sophisticated idea that assigns important functions to other neurochemical systems. While dopamine still plays a significant part in the "wanting" function, the opioid system likely plays a larger role in the "liking" function. The distinction of stimuli into pleasant/unpleasant (attractive/aversive) has been described in insects, and it is widely believed that this principle applies throughout the animal kingdom. Mushroom bodies have been identified as the critical regions of reward functions in the brains of insects, particularly in the two basic model species, the bee *Apis mellifera* and the fruit fly *Drosophila melanogaster*. The exact descriptions of the implicated neurotransmitters and modulators, as well as specific cellular and network structures, were also provided. Although the complexity of the brain networks in mammalian and insect reward systems differs (the mesolimbic dopaminergic network in mammals is composed of millions of neurons, whereas the mushroom body of insects has a number of cells that is probably two orders of magnitude lower), the general principles are the similar in both. The fly *Drosophila melanogaster* is a frequent laboratory model for investigating the principles of neural network functioning. When studying the brain reward system, it is not only appealing because it is a relatively simple organism with a transparent brain (its mushroom body has approximately 2200 Kenyon cells) and a described genome, but it may also have the benefit for us that when thinking about its brain, we do not apply relatively old, complex concepts with unlimited meanings. The complexity and limitless nature of concepts and functions is a difficulty in interpreting the study of the human brain. In the case of the fruit fly, we can highlight that 1) the brain regions involved in associative learning and brain reward functions are surprisingly complex, despite the fact that it is a relatively simple and short-lived organism, 2) its brain almost certainly has a system that

creates a motivational drive ("wanting" function), and 3) there are indications of the potential existence of a hedonic component of pleasure or its evolutionary predecessor, based not on endogenous opioids. It is inspiring in many ways to compare the brain structures of two evolutionary distinct animal groups—insects and mammals/humans. Several guiding ideas are visible in the reward system architecture: 1) the existence of multiple networks for various aspects of reward, 2) the hierarchy of networks, parallelism, and relative independence of individual reward processes, 3) the flow of information in the reward network occurs more as cyclical, reverberating activities than linearly, 4) dopaminergic neurons are a functionally diverse group, sometimes with specialized roles, and 5) developmentally different parts of the network are used in different ways. This comparison also has several implications for a broad paradigm of animal reward, including: Reward principles are universal, and all species are likely fundamentally motivated by the need for rewards. The brain reward mechanisms appear to be hierarchically structured; rather than being centrally organized, they are distributed among other brain networks and mechanisms. The components of these mechanisms can operate independently of one another and concurrently. While the function of neuropeptides in the reward system is flexible, the function of monoamines in the reward system is likely to be conservative in evolutionary terms (the function of endogenous opioids in mammals may be at least partially regulated by another neuropeptide in insects). The neurotransmitter identity of dopaminergic neurons in the reward system is likely to be very context-dependent and flexible. Two other interesting concepts can be found in the bee's reward system: the sublimation of reward functions in individuals in favor of collective (superorganismic) pleasure, and the implied integrated function connecting reward functions and social behavior into one continuum. The comparative study of different animals gives new scope for understanding disorders of the reward system, especially addiction, and may also have significant philosophical consequences.

## **Prohlášení**

Prohlašuji, že jsem autorem této disertační práce a že jsem ji vypracoval jen s použitím pramenů a literatury uvedených v seznamu citované literatury.

Jiří Dvořáček

Křenov

8. 10. 2023

Tato diplomová práce vznikla z partnerství Přírodovědecké fakulty Jihočeské univerzity a Entomologického ústavu Biologického centra AV ČR na podporu doktorského studia ve studijním programu Fyziologie a vývojová biologie.



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BIOLOGICKÉ  
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### **Finanční podpora**

Práce byla podpořena projektem RVO 60077344 Entomologického ústavu BC AV ČR.

### **Poděkování**

Chtěl bych poděkovat lidem z Entomologického ústavu BC AV ČR za trpělivost a podporu v průběhu všech let spolupráce. Zejména děkuji panu prof. Daliboru Kodříkovi za pečlivé vedení má práce, za nezištné sdílení svých vědeckých zkušeností, za lidsky velmi příjemný a podpůrný přístup, za inspirativní spolupráci u každého společného článku a za ukázkou toho, že věda se dá dělat poctivě.

## Seznam článků a autorského podílu

Tato práce je postavena na následujících článcích (chronologicky seřazených):

- I. **Dvořáček, J., & Kodrík, D. (2021).** Drosophila reward system - A summary of current knowledge. *Neuroscience & Biobehavioral Reviews* **123**, 301 - 351.  
<https://doi.org/10.1016/j.neubiorev.2020.12.032>

(IF = 9,052)

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- II. **Dvořáček, J., Bednářová, A., Krishnan, Natraj, Kodrík, D. (2022).** Dopaminergic mushroombody neurons in Drosophila: flexibility of neuron identity in a model organism? *Neuroscience and Biobehavioral Reviews* **135**, 104570.  
<https://doi.org/10.1016/j.neubiorev.2022.104570>

(IF = 8,2)

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- III. **Dvořáček, J., & Kodrík, D. (2022).** Drug effect and addiction research with insects - From Drosophila to collective reward in honeybees. *Neuroscience and Biobehavioral Reviews* **140**, 104816. <https://doi.org/10.1016/j.neubiorev.2022.104816>

(IF = 8,2).

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## **Souhlas spoluautora**

Prof. RNDr. Dalibor Kodrík, CSc., jako seniorní spoluautor všech uvedených článků plně potvrzuje autorský podíl Jiřího Dvořáčka u všech uvedených článků.



.....  
Prof. RNDr. Dalibor Kodrík, CSc.

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# **1. KAPITOLA**

## **Úvod**



## **1.1. Mozkový systém odměny jako univerzální nástroj k přežití živočichů**

Chování živočichů není náhodné, ale je dominantně vedeno k opakovanému dosahování libých momentů (odměny) a naopak k vyhýbání se momentům nelibým či ohrožujícím. Označení podnětů prostředí libostní hodnotou a posílení strategií vedoucí k zisku libého a vyhnutí se nelibému je funkcí mozkového libostního systému či systému odměny (v angličtině „reward system“). Základní funkce libostního systému je označovat biologicky významné podněty a situace (momenty podstatné pro přežití jedince a druhu, tedy zejména potravu a rozmnožování) libostní, hedonickou hodnotou, aby se zvýšila pravděpodobnost, že organizmus tyto biologicky významné momenty vyhledá i příště. Současně systém odměny vytváří predikci dosažení libosti a libostní hodnoty (např. Berridge & Kringelbach, 2008; Barron a kol., 2010).

Terminologická poznámka úvodem: pojem „odměna“ („reward“) lze používat v neurobiologii ve více blízkých významech, v tomto textu se budeme držet definice Berridge (2008): „positive affective value or hedonic impact of a reward stimulus“ a na některých místech pro snazší chápání budeme tento pojem volně zaměňovat za pojem „libost“. Stejně tak pro originální pojem „reward system“ budeme používat striktní překlad „systém odměny“ i v češtině jednoznačnější pojem „libostní systém“.

Systém odměny a celý model řízení chování potenciální libostí byl zprvu studován na savcích. Původní koncept savčího systému odměny byl soustředěn na mesolimbický dopaminergní systém sestávající hlavně ze dvou oblastí – z jader nucleus accumbens a ventrální tegmentální area. Klíčovou funkční strukturou podle této klasické představy je triáda složená z dendritického trnu GABAergních spiny neuronů v nucleus accumbens, z glutamatergních excitačních zakončení z prefrontálního cortexu a hippocampu a z inhibičních zakončení dopaminergních buněk z ventrální tegmentální arey. Dopamin na této synapsi moduluje kortikální a limbický tok informace do spiny neuronů. Dopamin v tomto modelu měl centrální roli a byl vnímán jako hedonický transmitter (Ikemoto a kol., 1997; Robinson & Kolb, 1997; Schultz, 1998; Spanagel & Weiss, 1999; Koob, 2000; 2004; Hyman, 2005; Kalivas & Volkow, 2005).

V současnosti je model savčího systému odměny komplexnější a dynamičtější. Jde spíše o soubor paralelních a částečně hierarchicky organizovaných funkčních okruhů, které zahrnují celé striatum (tedy včetně dorsálního), přední cingulární kortex, orbitální a dorsální prefrontální kortex, ventrální pallidum, amygdalu, hippocampus, thalamus, laterální habenulární nucleus a některé oblasti kmene (nucleus pedunculopontinus, nucleus raphe). Tyto části sítě se podílejí na mnoha dalších funkcích, ale společně hrají klíčovou roli v různých aspektech odměny a v různých momentech komplexní dynamiky zpracování odměny (Haber & Knutson, 2010; Castro & Berridge, 2014; Berridge & Kringelbach, 2015). Dopaminergní systém je nadále vnímán jako klíčový ale už není jediný: u některých aspektů odměny má dominantní úlohu systém endogenních opioidů (Berridge, 2009; Le Merrer a kol., 2009; Gosnell & Levine, 2009; Berridge & Kringelbach, 2013; 2015). U savců (a u člověka zvláště) jsou libostní funkce úzce spojeny s kognitivními operacemi jako je vytváření strategií k dosažení odměny, vytváření kognitivních reprezentací libosti nebo symbolických odměn –

libostní síť úzce spolupracuje s kognitivními oblastmi mozku (Haber & Knutson, 2010; Berridge & Kringelbach, 2013).

Libostní funkce, mozková odměna, jsou spíše komplexem více funkcionalit s různou dynamikou a různou fyziologickou rolí. Praktické je základní rozdělení libostních funkcí na komponentu “wanting”, “liking” a “learning” (Berridge, 2008; 2009; Berridge a kol., 2009; Berridge & Kringelbach, 2013; 2015). “Wanting” reprezentuje motivační drive a touhu získat libost, “liking” je vlastní hedonická komponenta a “learning” reprezentuje klasické a instrumentální podmiňování a vytváření kognitivních reprezentací libosti. U různých živočichů jsou tyto komponenty zastoupeny různě, v plné podobě u člověka/savců se “wanting” uplatňuje na začátku procesu získání odměny, “liking” je završením a konečným cílem, satietou v pravém slova smyslu, “learning” probíhá v průběhu celého procesu. Funkce “wanting” i “liking” jsou v mozku realizovány na různých místech a v evolučně různě starých oblastech od vývojově starších subkortikálních, kmenových oblastí po mladší kortikální, a je proto komplikované říci, že některá z těchto funkcí je vývojově mladší a jiná naopak evolučně pokročilejší (Berridge & Kringelbach, 2015). Samotný libostní proces může proběhnout i jen s komponentou “wanting” (např. u nižších živočichů nebo u libosti po požití stimulancí), či jen s komponentou “liking” (lze si představit libost bez drivu např. při jakékoli nečekané, neplánované odměně).

Pro funkci “wanting” je nadále klíčový zmíněný mesolimbický dopaminergní systém a nadále dopamin. Funkce dopaminu nejsou stále zcela jasné, pravděpodobně plní v libostním systému více rolí současně: dopamin může být nositelem samotné libosti (viz výše zmíněná původní hedonická hypotéza), dopamin může být signálem pro zahájení/posílení asociativního učení (“reward learning hypothesis”), může být nástrojem zaměření pozornosti či označení motivačního významu (“incentive salience hypothesis”), nástrojem pro predikci odměny (“prediction error theory”) nebo všechny tyto funkce dopaminu můžou být integrované (nejspíše v podobě, že různé skupiny dopaminergních neuronů mají různé výše zmíněné funkce (např. Rescorla & Wagner, 1972; Berridge, 2007; Schultz, 1997; 2006; Bissonette a kol., 2014; Cohen a kol., 2012; Bromberg-Martin a kol., 2010)).

Pro funkci “liking” se jeví být u savců jako klíčové endogenní opioidy. Generování vlastní hedonické složky či satiety je anatomicky koncentrováno do vcelku malých oblastí (označovaných jako hedonické hotspoty), které se nacházejí v libostním systému rozptýleně, hlavně ale v nucleus accumbens, ve ventrálním pallidu, v orbitofrontálním kortexu a insule a základním neurochemickým nástrojem zde jsou endogenní opioidy. Jejich stimulace vede ke generování vlastní libosti (Berridge, 2009; Le Merrer a kol., 2009; Gosnell & Levine, 2009; Berridge & Kringelbach, 2013; 2015).

Mozek nižších/bezobratlých živočichů je kvalitnější než jsme původně mysleli. Relativně nedávná představa byla, a) že hmyz funguje jako jednoduchý stroj bez složitějších mozkových funkcí, b) že hmyz a nižší živočichové vůbec dosahují přežití nikoliv zdokonalováním algoritmů chování, ale aplikací vrozených vzorů chování a c) že při velkém počtu jedinců v dané populaci tento vrozený vzorec chování s různou přesností odpovídá potřebám v daném životním kontextu a úprava těchto algoritmů se děje selekcí. Hmyz se umí ale učit. Umí

diskriminovat environmentální podněty na odměňující a averzivní, umí si je pamatovat a adaptovat podle toho své chování. Hmyz je schopen asociativního učení, přičemž klíčem, podle kterého se děje asociativní učení s vytvářením paměti, je odměna (lépe: atraktivní podnět) či trest (lépe: averzivní podnět), například v podobě sladká/hořká potrava. Atraktivní podnět pak vyhledává, averzivnímu podnětu se vyhýbá. V posledních letech bylo opakovaně popsáno, že tyto principy jsou podobné jako u savců/člověka a že funkce systému odměny je evolučně konzervativní (Perry & Barron, 2013; Waddell, 2013; de Araujo, 2016; Perisse a kol., 2016; Scaplen & Kaun, 2016; Cognini a kol., 2018; Kaun & Rothenfluh, 2017). Asociativní učení a mozkové struktury s tím spojené byly studovány na několika hmyzích modelech, např. na cvrčkovi *Gryllus bimaculatus* (např. Mizunami a kol., 2009; Terao a kol., 2014; Mizunami & Matsumoto, 2017), na čmelácích *Bombus* spp. (např. Palottini a kol., 2018), na parazitických vosičkách (např. Wäckers, 1994; Kruidhof a kol., 2012), na saranči *Locusta migratoria* (např. Perez -Orive a kol., 2002; Cassenaer & Laurent, 2007), na švábovi *Periplaneta americana* (např. Watanabe a kol., 2003), v této práci se ovšem budeme věnovat zejména dvěma modelům: octomilce *Drosophila melanogaster* a včele *Apis mellifera*.

## **1.2. Závislost jako porucha libostního systému**

Libostní systém není jen zajímavým předmětem studia vysvětlujícím principiální motivy chování živočichů a naše zacházení s životními radostmi a libostmi. Praktické implikace mohou plynout zejména z porozumění poruchám libostních systémů. Hlavní poruchou tohoto systému u člověka je závislost. Návykové látky (drogy) působí na mozkový libostní systém. V úvodní fázi mají odměňující efekt (kvůli kterému se zprvu užívají), ale opakovaním užívání dochází ke změnám v libostním systému a k vytváření jeho patologické rovnováhy. Původně dobrovolné užívání drogy se mění na kompulzivní. Drogy zvyšují v akutní fázi výdej dopaminu v oblasti shell v nucleus acumbens a vyvolávají odměnu (libost). Souběžně také probíhá asociativní učení, při kterém podněty spojené s užitím drogy jsou asociovány a po určité době začnou samy vyvolávat aktivaci libostního systému jako prediktor odměny/libosti (Wise, 2008; Volkow & Morales, 2015; Di Chiara, 2002; Koob, 1998; Schultz, 2002). Jednotlivé drogy se liší mechanismem, jakým hladinu dopaminu zvyšují: stimulancia zvyšují extracelulární hladinu dopaminu zejména blokováním presynaptického dopaminergního transportéru (DAT), který ze synaptické štěrbině za normálních okolností vychytává dopamin zpět do zakončení presynaptického neuronu. Další drogy, jako např. opioidy nebo alkohol, zvyšují extracelulární dopamin nepřímo inhibicí GABA neuronů ve ventrální tegmentální aree (Hayes a kol., 2020). Souběžně drogy (hlavně opioidy a alkohol) působí na  $\mu$  a delta opioidní receptory v libostních oblastech a vyvolávají zde vlastní hedonickou reakci (Colasanti a kol., 2012; Le Merrer a kol., 2009; Murphy, 2015).

Při opakovaném užívání se situace začíná měnit. V libostním systému dochází k neuroadaptacím různého typu (transmitterových, synaptických, receptorových, druhých poslů, časných genů atd.), klesá senzitivita dopaminergních buněk na odměnu (rozvíjí se tolerance), zvyšuje se reaktivita na stres a dochází postupně i k poškození některých neuronálních struktur (např. užší a méně funkční dendritické trny v nucleus accumbens (Russo a kol.,

2010)). Užívání drogy je již motivováno především odstraněním nežádoucích efektů, dochází k přesmyku ke kompulzivnímu užívání, k distorzi původní libostní funkce a také ke změnám ve vyšší kontrole libostních funkcí (Koob & Volkow, 2010; Uhl a kol., 2019; Volkow a kol., 2016; 2011; Hayes a kol., 2020). Komplexní, a pravděpodobně paralelně probíhající, neurobiologické mechanismy rozvoje závislosti popisují tři dominantní teorie (kterým se nyní blíže věnovat nebudeme): teorie pobídkové významnosti - „incentive salience theory“ (Robinson & Berridge, 1993; 2008), teorie formování návyku – „habit hypothesis“ (Everitt & Robbins, 2005) a koncept allostázy (Koob & Le Moal, 2005).

Tyto změny libostního systému jsou sice u závislostí univerzální a klíčové, závislost je ovšem komplexní problém s poruchou mnoha mozkových a somatických systémů a s komplexní patologií hodnot a celého životního kontextu člověka. Tato komplexita vedla k mnoha různým přístupům k závislosti a k mnoha různým teoriím vystihujícími vesměs jen některé z mnoha aspektů problému a používajícími navíc mnohdy nekompatibilní pojmy. Z řady důvodů se jeví jako výhodné při studiu libostního systému i studiu závislosti využívat k pochopení jejich neurobiologické podstaty co nejjednodušší zvířecí modely.

### **1.3. Nezbytnost komparativního pohledu**

Mozek člověka je mimořádně komplexní soubor neuronálních sítí, které jsou navzájem složitě propojeny, jeho architektura se mění v čase v důsledku zrání/stárnutí, v důsledku životních zkušeností a průběžných či dramatických změn životního kontextu. Průběžně nedochází jen ke změně mozkové struktury (zejména propojení), ale flexibilně se mění i neurochemické parametry v závislosti na aktivitě neuronů a na kvalitě prostředí/širšího kontextu (Brezina, 2010; van Praag a kol., 2000; Sale a kol., 2014; Spitzer, 2015). Mozkové funkce nejsou navíc pravděpodobně v mozkové struktuře organizovány modulárně (koncept modularity předpokládal, že konkrétní funkcionalita mozku je spojená s konkrétní strukturou, a tedy že popisem struktury pochopíme i funkci), ale mozkové funkce mohou být distribuovány ve více typech sítí současně, pokaždé jinak, a naopak: konkrétní mozková struktura může být využívána („reuse“) více funkcemi, pokaždé pravděpodobně jinými (Anderson, 2010; Brezina, 2010; Marder, 2011). Tato funkční univerzalita neuronálních sítí znemožňuje pochopení mozku pouhou statickou deskripcí. Mozek člověka je navíc komplexem sítí víceúrovňových, zčásti hierarchicky organizovaných, kvalita celého mozku nemůže být pochopena z porozumění funkce struktury či jedné úrovně struktur (Bargmann and Marder, 2012). Ve snaze o pochopení neurobiologických principů našeho mozku nutně musíme využívat přehlednější, čitelnější a jednodušší nervové systémy, kde aspekt komplexity a vyšších systémových úrovní není pravděpodobně tak důležitý.

Pochopení funkce mozkových funkcí je komplikováno i jazykovým problémem.

Neurobiologická úroveň používá jiný jazyk než úroveň psychologická (klinická) a obě jazykové úrovně jsou velmi obtížně kompatibilní. Neurobiologické pojmy jsou přesnější, významově ohraničenější a jednoznačné (např. neuron, synapse, dopamin...), nejsou ale schopné pojmut širší kontext a obvykle nejsou schopné vysvětlit mozkovou funkci na úrovni

naši běžné lidské zkušenosti. K tomu jsou používány pojmy komplexnější, vícevýznamové z úrovně psychologické (klinické). Tyto pojmy jsou ovšem velmi často dnes již významově neohraničené, mnohdy je jejich původ starověký (např. základní pojmy pro popis duševních funkcí – emoce, pocity, vědomí...), v průběhu jejich požívání v historii nabyly různých významů a jsou nevyužitelné pro popis mozku na neurobiologické úrovni nejen pro svou nejednoznačnost, ale také proto, že interpretace neurobiologických prací ovlivňují svými vedlejšími či komplexními významy. Využití jednodušších zvířecích modelů v neurobiologii (ne-obratlovců) může tuto situaci usnadnit, neboť méně často nás na zkoumání mozku např. hmyzu napadne aplikovat pojmy psychologické.

Hlavní funkcí mozku a všech nervových soustav živočichů je - kromě řízení a koordinace většiny funkcí organismu – adaptace na vnější životní podmínky, které mohou být velmi složité a jsou v různé míře proměnlivé. V tomto smyslu mozek a prostředí tvoří kontinuum, ve kterém jen s velkými omezeními lze sledovat plnou funkci mozkových funkcí. Člověk má životní kontext velmi komplikovaný a dynamicky se měnící, při studiu vztahu konkrétní mozkové funkce (v našem případě libosti) a prostředí nelze spolehlivě určit, který z mnoha parametrů aktuálního kontextu je klíčový. Člověk také patří mezi dlouho žijící živočichy a zachytit tak dlouhodobou dynamiku (ontogenezi i proměny v průběhu dospělého života) dané mozkové funkce laboratorně nelze. Stejně tak lze komplikovaně sledovat u člověka aspekty evoluční (konkrétní podoba mozkové funkce je mimo jiné výsledkem evolučních adaptací a selekcí). Kontinuum mozek-prostředí je snadněji sledovatelné u krátce žijících organismů s krátkým vývojem, u kterých lze podmínky prostředí regulovat, redukovat a sledovat během více generací.

Při zkoumání mozkových funkcí člověka jsme často zatíženi antropocentrismem – máme tendenci naše mozkové funkce vnímat jako nejdokonalejší a jedinečné. Je nutné ovšem připustit, že v živočišné říši vyšší duševní funkce nejsou vždy tím nejdokonalejším nástrojem adaptace, nervové systémy jednotlivých druhů mají jedinečné designy a jedinečné funkce, které odpovídají nejvíce jejich evolučním potřebám. Pro pochopení jedinečnosti konkrétní mozkové funkce konkrétního druhu (i člověka) je komparace s jinými živočichy nezbytná – v podobě zkoumání živočichů i evolučně vzdálených (komparativní neurobiologie).

Tématem práce je studium mozkového libostního systému hmyzu („brain reward system“) a komparace základních principů fungování tohoto systému hmyzu s libostním systémem savců/člověka. Cílem bylo zodpovědět některé dílčí otázky: Můžeme libostní funkce u hmyzu skutečně považovat za ekvivalentní těm, které známe u savců/člověka? Má mozek hmyzu dostatečnou kvalitu pro přítomnost hedonické složky libosti – pro komponentu “liking”? Jsou neurochemické podmínky mozkové odměny podobné savčím/lidským nebo jsou odlišné? Jsou homologie dostatečné natolik, abychom z nich mohli čerpat inspiraci pro neurobiologii člověka? Jestliže ano, lze z těchto homologíí libostního systému vyvodit obecné principy fungování živočišné libosti? Cílem srovnání dvou evolučně vzdálených typů organizace libostních systémů je snaha jednak formulovat obecné principy libosti u všech živočichů, a také nabídnout nové pohledy na řízení libosti u člověka a na mechanismus vzniku některých poruch s tímto mozkovým systémem spojených.

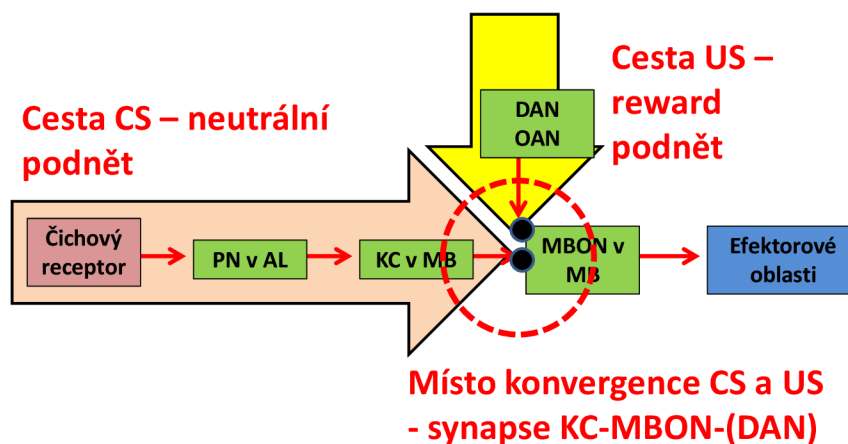
Práce je zpracována v podobě třech samostatně publikovaných textů. První text zevrubně shrnuje současné informace o asociativním učení u octomilky a zaměřuje se na přítomnost strukturálních a funkčních podmínek pro přítomnost jednotlivých složek libosti. Druhý text se věnuje neurotransmitterové identitě dopaminergních neuronů u savců a u octomilky, diskutuje význam ko-transmise v těchto libostních strukturách, neurotransmitterového switchingu a flexibility libostního systému v závislosti na environmentálním kontextu u savců i octomilky. Třetí text se zaměřuje na libostní systém včel a na experimenty s působením drog na tento libostní systém a diskutuje srovnání s působením drog na libostní systém savců/člověka,

# **2. KAPITOLA**

## **Shrnutí výsledků**

V neurobiologickém výzkumu jsou používány zejména dva hmyzí modely – octomilka *Drosophila melanogaster* a včela *Apis mellifera*. Oba druhy mají v organizaci systému odměny mnoho podobností, ale zároveň některé jedinečnosti, a liší se ve stupni mozkové komplexity (přibližně 100 000 neuronů octomilky proti 1 000 000 včely, resp cca 2 500 neuronů mushroom body octomilky proti 300 000 včely (Witthöft, 1967; Raji & Potter, 2021; Menzel, 2012). Základní neurofyziologické principy jsou čitelnější u *Drosophily*, včela může být atraktivní schopností vyšších forem učení (Giurfa, 2003; Menzel, 2012; Devaud a kol., 2015) a podstatnou sociální složkou libosti – tedy momenty inspirativními pro studium libosti lidské.

Základní strukturou systému odměny *Drosophily* (=základní strukturou asociativního učení) je místo konvergence informace o neutrálním podnětu (typicky vůně) a informace o libostní/averzivní hodnotě (**obrázek 1**): synapse axonu Kenyonovy buňky, mushroom body output neuronu a dopaminergního (resp. octopaminergního) neuronu (např. Schwaerzel a kol., 2003; Riemensperger a kol., 2005; Busto a kol., 2010; Waddell, 2010). U včely asociativní učení probíhá na více úrovních mushroom body a na rozdíl od zpracování hlavně čichové informace jde o informaci multisenzorickou, další principy jsou ovšem podobné (Farooqui a kol., 2003; Giurfa, 2003; Haenicke a kol., 2018; Szyszka a kol., 2008).



**Obrázek 1:** Základní funkční struktura systému odměny *Drosophily*

Synapse mezi axony Kenyonových buněk (KC) a mushroom body output neuronu (MBON), kde je čichová informace o neutrálním podnětu (CS – podmíněný podnět) jdoucí z čichového receptoru cestou projekčních neuronů (PN) v antenálním lobu (AL) do Kenyonových buněk v mushroom body (MB) asociovaná s libostní hodnotou (US – nepodmíněný podnět) reprezentovanou aktivitou dopaminergních (DAN), případně octopaminergních neuronů (OAN).

Síť mushroom body, zapojená do asociativního učení a do základního libostního označování stimulů „atraktivní/averzivní“, je ovšem složitější než je zmiňovaná triáda. Mushroom body



Drosophily je tvořena cca 2 200 Kenyonových buněk 7 typů, 34 mushroom body output neurony 21 typů (cholinergními, glutamatergnými, GABAergními), 130 dopaminergními neurony 20 typů (clustery PAM a PPL1) a několika zpětnovazebnými neurony (Aso a kol., 2014a; 2014b; Ichinose a kol., 2015; Felsenberg a kol., 2017; Cognini a kol., 2018). Mezi těmito buňkami je mnoho typů vesměs asymetrických propojení. V organizaci této sítě Drosophily lze vidět – alespoň v náznacích - podobné principy vnitřní architektury jako u systému odměny savců/člověka: 1) přítomnost více sítí pro různé aspekty odměny, 2) hierarchické uspořádání sítí, paralelnost a současná relativní nezávislost jednotlivých libostních procesů, 3) tok informace v libostní síti se odehrává spíše v podobě cyklických, reverberujících aktivit než lineárně, 4) dopaminergní neurony jsou funkčně pestrou skupinou, někdy se specializovanými rolemi, 5) v libostní funkci se uplatňují vývojově různě staré části sítě (Aso & Rubin, 2016; Takemura a kol., 2017; Aso a kol., 2014a; Keene a kol., 2006; Thum & Gerber, 2019).

Z komparativního studia libostního systému člověka a hmyzu lze vyvodit několik důsledků a možných inspirací pro další zkoumání.

### 1. Malý mozek může mít kvalitní funkci

Běžnou představou je, že ke kvalitní mozkové funkci je potřeba většího mozku. Ukazuje se však, že i miniaturní mozek hmyzu s velmi omezeným počtem neuronů je schopen asociativního učení. Malý mozek má samozřejmě vícero nevýhod daných převážně fyzikálními limity, např. větší spotřeba energie vzhledem k většímu poměru mozku k tělu než u větších živočichů, méně neuronů (neurony mají limity pro miniaturizaci, molekulární struktury miniaturizovat od určité úrovně nelze), pomalejší hodnotu přenosu informace (rychlost přenosu je mimo jiné úměrná průměru axonu), větší riziko vzniku informačního šumu, méně propojení, omezení v paralelním zpracování atd. Mozek hmyzu dokázal ovšem najít evoluční „triky“, jak při malé velikosti sítě zachovat co nejlepší funkci, v našem případě asociativní učení (např. rozpuštění těl neuronů u dospělce a sestavení sítě v podstatě jen z výběžků nebo větší funkční univerzalita neuronálních prvků – důsledný „reuse“ (Polilov, 2012; Niven & Chittka, 2010).

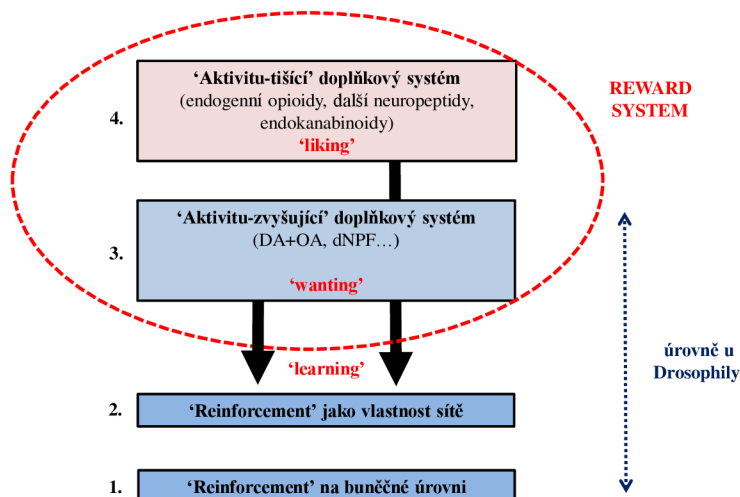
### 2. Libostní principy jsou univerzální – usilování o libost je pravděpodobně základní vlastností všech živočichů

Existence systému odměny v mozku hmyzu je zřetelně doložena, minimálně na úrovni komponenty “wanting” s tím, že jsou zaznamenány funkce neuropeptidů, které imponují základním prostředím pro vývoj funkce “liking” (např. Shohat-Ophir a kol., 2012; Shao a kol., 2017; Kaun a kol., 2011; Devineni & Heberlein, 2009). Uvážíme-li schopnost asociativního učení u jednodenních larev Drosophily (Eichler, 2017) či u háďátka *Caenorhabditis elegans* s malým počtem neuronů (Amano & Maruyama, 2011; Rahmani & Chew, 2021), můžeme uzavřít, že pro fungování bazální libostní funkce není potřeba složitě nervové sítě.

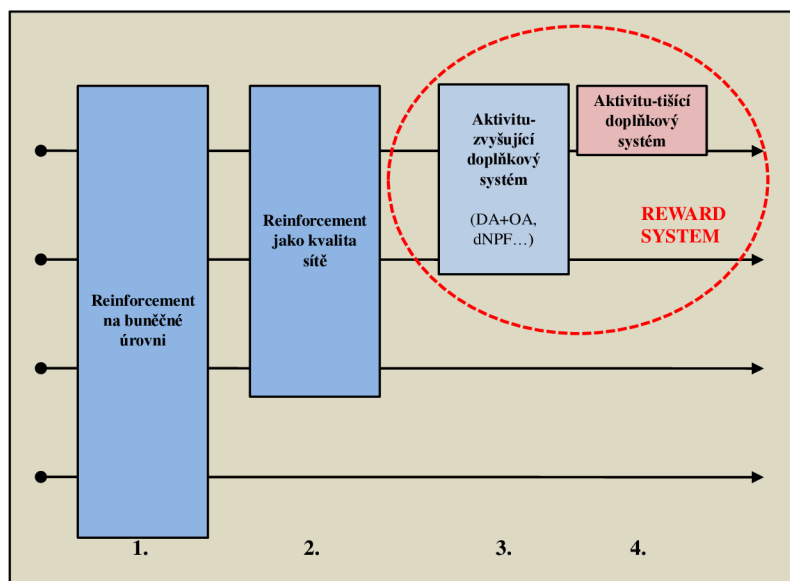
Libostní funkce se jeví být další evoluční vrstvou v hierarchii spojení konkrétního podnětu prostředí s odpovídající reakcí živočicha na tento podnět. Hypoteticky si lze představit tuto hierarchii (**obrázek 2**) posílení spojení chování s podnětem (“reinforcement”):

- Buněčná úroveň – i na úrovni jedné buňky lze doložit existenci minimální volby mezi “žádoucím” a “nežádoucím” chováním buňky v reakci na konkrétní parametr prostředí - viz např. experimenty s bakterií (Koshland, 1980), se spermií (Eisenbach, 2004) nebo s neuronem (Debanne a kol., 2019).
- Síťová úroveň – posílení může být obecnou vlastností sítě v podobě reverberace informace, synchronních oscilací neuronů či Hebbianského učení.
- “Aktivitu-zvyšující doplňkový systém” – existence síťového prvku, který reakci na konkrétní podnět zvýhodní před reakcí na jiné podněty, např. tím, že sníží zaměření pozornosti na jiné podněty, označí aktuální podnět jako významnější, vymezi pro zpracování aktuálního podnětu další části sítě. V našem případě jde o systémy monoaminů, zejména dopaminu, z hlediska libosti se projevující jako funkce “wanting”. Tuto úroveň posílení lze jednoznačně nalézt i u hmyzu.
- “Aktivitu-tišící doplňkový systém” – doplňuje předchozí systém o dosažení konečné satiety a utišení aktivity jiných systémů (alertních, algických, stresových, motorických, senzorických). Tento systém se pravděpodobně vyvinul z některých neuropeptidových systémů, které tišily aktivitu po dosažení homeostatického či konkrétního metabolického cíle. Endogenní opioidy s generalizovaným tišícím efektem na všechny zmíněné systémy vyvolávají generalizovanou satietu (meta-satiety) jako nejvyšší formu libostní komponenty “liking”. Plně vyvinutá komponenta “liking” by měla přechodně tišit i předcházející komponentu “wanting” (tedy např. jsou diskutabilní ty metodiky sledující atraktivitu drogy u modelových živočichů podle zvýšené motorické aktivity). Neurochemické prostředí meta-satiety (tedy přítomnost endogenních opioidů) je popsáno zatím jen u obratlovců, dílčí aktivitu-tišící prvky zprostředkované jinými neuropeptidy ale zaznamenané jsou už i u hmyzu.

Tyto jednotlivé úrovně pracují pravděpodobně paralelně a částečně nezávisle na sobě. K posilování spojení podnět-reakce může docházet u vyšších živočichů na všech zmíněných úrovních (buněčné, síťové, aktivitu-budící systémy, aktivitu-tišící systémy). Lze předpokládat, že i „jen“ na úrovni buněčné a síťové dochází každodenně k obrovskému množství různě významných posílení, většina spojení podnět-reakce se může odehrávat bez potřeby asociativního učení a bez použití systému odměny (**obrázek 3**). Hypoteticky můžeme předpokládat, že různé poruchy typu závislosti se můžou odehrávat i na těchto úrovních či minimálně všechny tyto úrovně jsou do vzniku poruchy zapojeny.



**Obrázek 2:** Základní úrovně posílení spojení podnět-reakce u živočichů od buněčné úrovně až po meta-satiету.

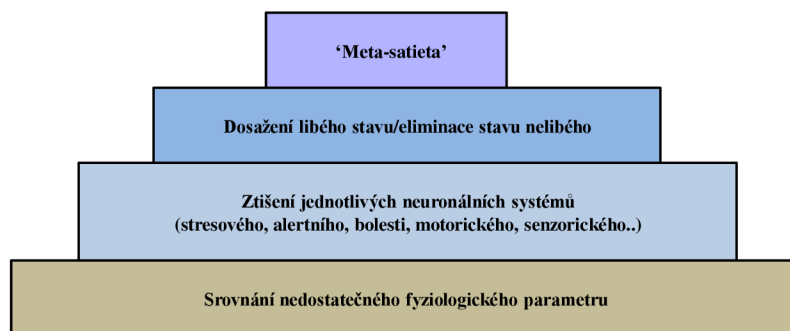


**Obrázek 3:** Paralelnost a nezávislost jednotlivých úrovní posílení reakce na podnět

Jednotlivé úrovně posílení spojení podnět-reakce u živočichů pracují paralelně i nezávisle. I u člověka se posílení vyskytuje na všech úrovních, k posílení některých reakcí bezesporu stačí úrovně buněčné a zejména síťové, u jiných stačí funkce “wanting” bez satiety, jiné jsou posíleny satiетou. Hierarchie úrovní posílení nemusí znamenat, že vyšší úrovně jsou silnější než nižší.

### 3. Psychologické fenomény jsou v základu fyziologické.

Projevy libostního systému u člověka a poruchy tohoto systému jsou často popisovány ryze psychologickými teoriemi a pojmy (např. libido, slast, závislost jako reakce na vnitřní konflikt, závislost jako důsledek nezvládnutého procesu individuace atd.). To může imponovat dojmem, že jde o ryze psychologický fenomén existující nezávisle na fyziologické podstatě. Existence společných libostních principů systému odměny u hmyzu, u kterého bychom tyto pojmy a teorie nepoužili, ukazuje zřetelné fyziologické jádro. Mozková odměna u člověka má komplexní psychologickou podobu, ta je ale zřejmě nejvyšší úrovní stojící na základních úrovních fyziologických. **Obrázek 4** ukazuje možné úrovně zklidnění na cíl zaměřené aktivity – od základní úrovně spočívající v harmonizaci nedostatečného konkrétního fyziologického parametru přes ztišení aktivit jednotlivých neuronálních systémů a přes dosažení libých stavů až ke generalizované meta-satietě, u které budeme vnímat spíše symbolické a psychologické významy než původní významy fyziologické, ze kterých se ale postupně pravděpodobně vyvinula.

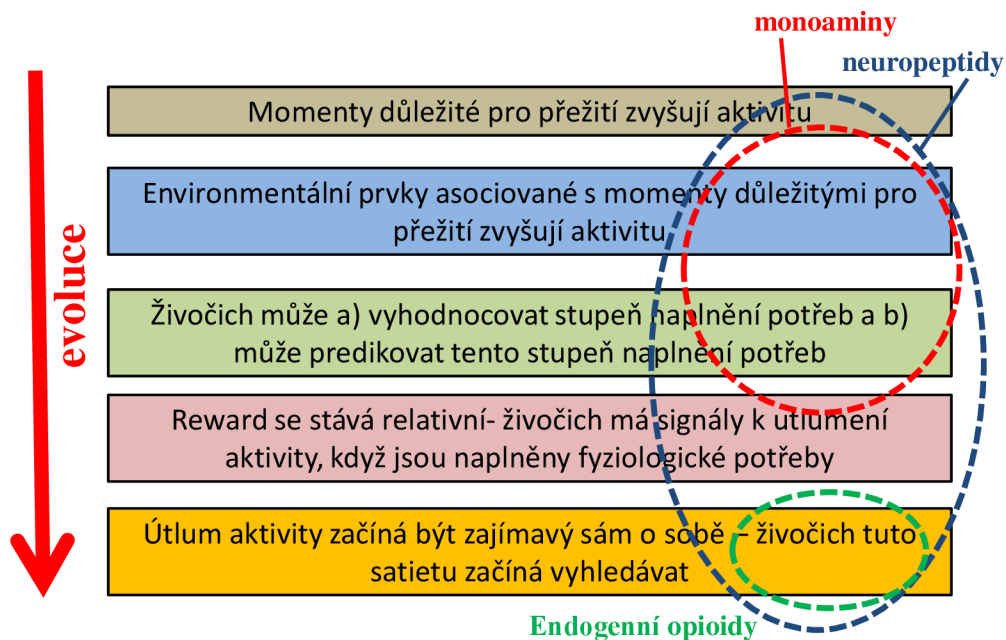


**Obrázek 4:** Možné úrovně zklidnění na cíl zaměřené aktivity.

### 4. Konzervativní libostní funkce monoaminů, flexibilní funkce neuropeptidů (obrázek 5)

Srovnáním funkce monoaminů u jednotlivých hmyzích modelů a mezi hmyzem a savci/člověkem se zdá být zřejmé, že funkce monoaminů (zejména dopaminu) je u živočichů v libostních systémech evolučně konzervativní. Dopamin má v libostních funkcích pravděpodobně více souběžných funkcí, ty jsou ale téměř identické u hmyzu i u savců (Perry & Barron, 2013; Waddell, 2013; Perisse a kol., 2016; Scaplen & Kaun, 2016; Cognini a kol., 2017). Naopak funkce neuropeptidů se jeví být vývojově flexibilní. Různé neuropeptidy

vykazují stejné či podobné funkce, funkce “liking” nemusí být nutně svázána s rolí endogenních opioidů, jako je tomu u savců, ale mohou ji u jiných živočichů reprezentovat jiné neuropeptidy, u hmyzu “liking”-like funkci vykazuje například allatostatin A (Hergarden a kol., 2012; Chen a kol., 2016) a neuropeptid F (Root a kol., 2011; Krashes a kol., 2009). U hmyzu je dobře popsána role neuropeptidů v nastavování hodnoty libosti podle metabolických potřeb organismu (např. Tsao a kol., 2018) – tato funkce u savců/člověka zatím příliš zkoumána není.



**Obrázek 5:** Role monoaminů a neuropeptidů v různých evolučních úrovních odměny

##### 5. Pravděpodobně univerzální flexibilita transmiterů v libostních oblastech

Při popisu mozku živočichů stále dominuje statický přístup: popisuje se co nejpřesněji anatomická struktura a předpokládané dráhy pro toky informací, identita neuronu je pak fixně dána neurotransmiterem, který obsahuje (Eccles a kol., 1954). Neurotransmiteru se připisuje centrální místo ve snaze porozumění funkci. Plasticita je chápána jako obměna struktur, které jsou jinak pevně dány. V dnešní době je již dostatečně doloženo, že ko-transmise více transmiterů (nejen transmiteru a modulátoru, ale i více “fast-acting” transmiterů současně) je normou a že neuron mění svou transmiterovou identitu podle potřeb sítě (neurotransmiterový switching (Spitzer, 2015)). Pokud jde o libostní síť, můžeme shrnout:

- Ko-transmise v dopaminergních neuronech v savčí ventrální tegmentální aree je funkčně nezbytná, pro libostní funkce je důležitá subpopulace duálních neuronů (exprimujících kromě dopaminu i glutamát) v tomto jádře (např. Root a kol., 2016; Greengard, 2011). Ko-transmise v dopaminergních neuronech v mushroom body *Drosophily* byla popsána také zatím bez jasného funkčního významu (Croset a kol., 2018; Nässel, 2018; Takemura a kol., 2017).
- Počet neuronů s dopaminovým fenotypem je activity-dependentní a mění se podle environmentálních podmínek (např. Dulcis a kol., 2013; Dulcis & Spitzer, 2008; Spitzer, 2017). Flexibilita neurotransmiterového vybavení v závislosti na životním kontextu ruší ideu statického mozku nezávislého na prostředí a zavádí ideu funkčního kontinua mozek-prostředí (mozek se pružně mění podle životního kontextu – v různých etapách života různě pružně).

## 6. Kolektivní odměna včel jako inspirace pro odměnu u člověka

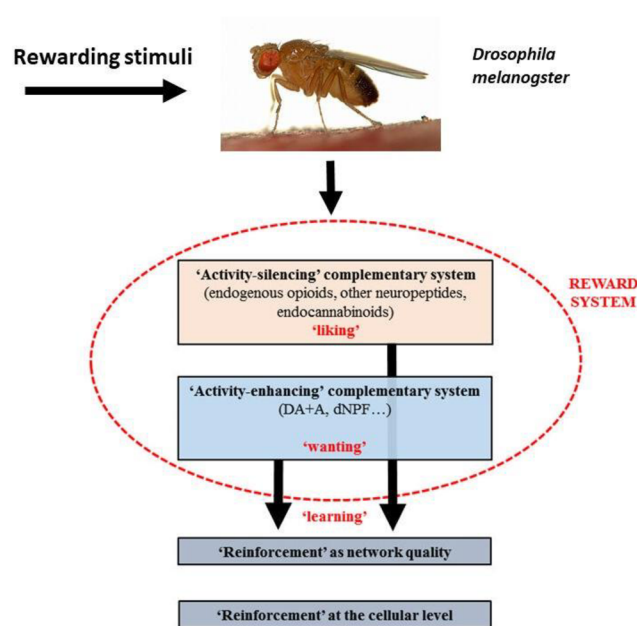
Včely jsou dle všeho schopné vyšších forem učení, které vykazuje některé stejné znaky jako učení obratlovců (např. kontextuální učení, kategorizace, učení se abstraktním pravidlům, schopnost učit se vztahu mezi dvěma stimuly (Giurfa, 2003; Menzel, 2012; Avergues-Weber & Giurfa, 2013; Devaud a kol., 2015)), a mají zárodky kapacit pro subjektivní zkušenost (schopnost tvořit environmentální reprezentace subjektivně a "egocentricky", (Barron & Klein, 2016)). Z hlediska funkcí libosti je ale zajímavější jejich aspekt sociální. Včelí kolonie se v mnoha aspektech chová jako organizmus, tvoří evolučně nový typ společenstva - superorganizmus, ve kterém výkon a dovednosti kolonie jsou vyšší než suma individuálních včelích dovedností (Moritz & Fuchs, 1998; Canciani a kol., 2019; Bernadou a kol., 2021). Kolektivní učení a rozhodování má také vyšší kvalitu než u individuálních včel, ale překvapivá je distribuce libostních funkcí. Libostní funkce jsou v kolonii distribuovány nerovnoměrně, každá kasta se konkrétním způsobem podílí na reward chování celé kolonie, ale "nikdo nemá všechno". Dělnice mají dobře vyvinuté asociativní učení, ale jejich aktivita nevede k individualní odměně. Královna, která nastavuje reward setting celé kolonie, nesměruje k získání odměny, její chování je ovlivňováno jinými parametry (např. densitou dělnic), královna zřejmě nemá vyvinuté asociativní učení, její mushroom body je velmi malé (Moritz & Fuchs, 1998; Roat & da Cruz Landim, 2008; Alaux a kol., 2010; Johnson & Frost, 2012; Muenz a kol., 2015). Ve včelí kolonii došlo pravděpodobně k sublimaci individuální odměny a je proto lepší mluvit o superorganizmické nebo kolektivní odměně a studovat ji na úrovni celé kolonie. Toto rozpuštění individuální odměny ve prospěch libosti celé kolonie může být zajímavou inspirací pro studium libosti u některých vysoce organizovaných, komplexních lidských společenstev (např. sekt či totalit).

## 7. Mezi libostními funkcemi a socialitou může být kontinuum

Vztah libosti a socializace včel může dávat další zajímavé inspirace. Kolektivní odměna může být manifestací komplexního sociálního chování. Někteří autoři popisují roli mushroom body v kontrole sociálních interakcí (např. Paffhausen a kol., 2020), jiní zvažují vyvinuté mushroom body jako pre-adaptaci pro rozvoj sociality (Farris, 2016). Podobně u obratlovců popisuje O'Connell & Hofmann (2010) "social decision-making network" - integrovanou síť spojující funkce odměny a sociálního chování a vytvářející funkční kontinuum. Neuroanatomická blízkost odměny a sociality naznačuje možný širší funkční systém, který u některých druhů má více tvar funkcí odměny, u jiných tvar sociálního chování.

# 3. KAPITOLA

## Článek 1 - Systém odměny *Drosophily* – shrnutí současných znalostí



Dvořáček, J., & Kodrík, D. (2021). *Drosophila* reward system - A summary of current knowledge. *Neuroscience & Biobehavioral Reviews* **123**, 301 - 351.  
<https://doi.org/10.1016/j.neubiorev.2020.12.032>





## Drosophila reward system - A summary of current knowledge

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### ARTICLE INFO

#### Keywords:

Reward system  
Dopamine  
Endogenous opioids  
Mushroom body  
Drosophila

### ABSTRACT

The fruit fly *Drosophila melanogaster* brain is the most extensively investigated model of a reward system in insects. *Drosophila* can discriminate between rewarding and punishing environmental stimuli and consequently undergo associative learning. Functional models, especially those modelling mushroom bodies, are constantly being developed using newly discovered information, adding to the complexity of creating a simple model of the reward system. This review aims to clarify whether its reward system also includes a hedonic component. Neurochemical systems that mediate the 'wanting' component of reward in the *Drosophila* brain are well documented, however, the systems that mediate the pleasure component of reward in mammals, including those involving the endogenous opioid and endocannabinoid systems, are unlikely to be present in insects. The mushroom body components exhibit differential developmental age and different functional processes. We propose a hypothetical hierarchy of the levels of reinforcement processing in response to particular stimuli, and the parallel processes that take place concurrently. The possible presence of activity-silencing and meta-satiety inducing levels in *Drosophila* should be further investigated.

## 1. Introduction

### 1.1. Parameters of the brain reward system were formulated first in the mammalian brain

In recent years, insect neurobiological research has suggested that insects also have a brain reward system with similar principles of functioning as mammals/humans. The reward system of insects began to be studied only after many important studies were conducted regarding the reward system of mammals, including humans. It can be generalized that the reward systems of all animals show fundamental similarities and that these key principles of structure and function of the reward system are evolutionarily conserved (Perry and Barron, 2013; Waddell, 2013; de Araujo, 2016; Perisse et al., 2016; Scaplen and Kaun, 2016; Cognini et al., 2018; Kaun and Rothenfluh, 2017).

It is necessary to make a distinction between two terms -

reinforcement and reward; studies rarely distinguish these two different terms. Berridge (2008) highlights the confusion surrounding the concept of reinforcement, which has several definitions: 1) 'positive affective value or hedonic impact of a reward stimulus', 2) 'associative strengthening of learned stimulus-stimulus or stimulus-response links without any affective connotation', 3) 'an observed strengthening of prior responses on which the reinforcer is contingent with no explanatory connotations.' Although studies of the reward system usually do not distinguish these meanings, in this article, the word 'reward' is used to represent definition 1, and the word 'reinforcement' for definitions 2 and 3.

The basic parameters of the function and significance of the reward system were determined by studies of the mammalian brain. The original concept of the mammalian reward system focused on the mesolimbic dopaminergic system consisting primarily of two brain regions — the nucleus accumbens (NAc) and the ventral tegmental area (VTA). The

**Abbreviations:** Ach, acetylcholine; AL, antennal lobe; APL, anterior paired lateral neuron; CBR, cannabinoid receptor; CRE, crepine neuropil; CS, conditioned stimulus; DA, dopamine; DAN, dopamine neuron; dDAT, *Drosophila* dopamine transporter; DMS, dorsal medial striatum; dNPF, *Drosophila* neuropeptide F; dopR, dopamine receptor; DPM, dorsal paired medial neuron; GABA, gammaaminobutyric acid; glu, glutamatergic MBON; Glu, glutamate; HT, serotonin; ILP, insulin-like protein; KC, Kenyon cell; LH, lateral horn; LTM, long-term memory; MB, mushroom body; MBON, mushroom body output neuron; NAc, nucleus accumbens; NPF, neuropeptide F; OA, octopamine; OAN, octopamine neuron; OFC, orbitofrontal cortex; PAM, protocerebral anterior medial cluster; PER, proboscis extension reflex; PFC, prefrontal cortex; PN, projection neuron; PPL, protocerebral posterior lateral cluster; SIP, superior intermediate protocerebrum; SLP, superior intermediate protocerebrum; SMP, superior medial protocerebrum; SNc, substantia nigra pars compacta; sNPF, short neuropeptide F; STM, short-term memory; TH-VUM, dopaminergic ventral unpaired median neuron; US, unconditioned stimulus; VTA, ventral tegmental area.

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<https://doi.org/10.1016/j.neubiorev.2020.12.032>

Received 5 May 2020; Received in revised form 16 December 2020; Accepted 27 December 2020

Available online 6 January 2021

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presumed key functional structure was a triad composed of the dendritic spine of the GABAergic spiny neuron in the NAc, the glutamatergic excitatory termination from the prefrontal cortex and the hippocampus, and the inhibitory termination of dopamine cells from the VTA, where dopamine (DA) modulates cortical or limbic glutamatergic input into GABAergic medium spiny neurons (Ikemoto et al., 1997; Robinson and Kolb, 1997; Schultz, 1998; Spanagel and Weiss, 1999; Koob, 2000; Nicola et al., 2000; Esch and Stefano, 2004; Hyman, 2007; Kalivas and Volkow, 2005).

At present, the mammalian reward system is viewed in a much more complex and dynamic form. The original idea that reward-guided learning is mediated only by dopaminergic VTAs that direct information to the nucleus accumbens has been replaced by a model of parallel and hierarchically organized functional circuits of the entire striatum (including dorsal), anterior cingulate cortex, orbital prefrontal cortex and ventral pallidum, and other interconnected areas such as the dorsal prefrontal cortex, amygdala, hippocampus, thalamus, lateral habenular nucleus and some brain stem regions (nucleus pedunculopontinus, nucleus raphe). These areas form a complex neural network that conveys various aspects of reward (Haber and Knutson, 2010; Castro and Berridge, 2014; Berridge and Kringelbach, 2015). Dopaminergic neurons (especially the ventral striatum) continue to play a key role, but more in the reward motivational component of reward rather than in hedonic reaction itself (Berridge and Kringelbach, 2015).

A brief summary of the organization of the reward system of mammals should help recall some principles of network organization, which could be found, or at least indicated, in the reward system of insects and are also mentioned in other parts of this text: 1) existence of multiple concurrent networks for different aspects of reward, 2) hierarchical arrangement of reward networks, parallelism and apparently relative independence of individual reward processes, 3) information flow in a hierarchized network of reward systems that do not happen linearly, but rather in the form of cyclic, reverberating activities, 4) that dopaminergic neurons are a structurally and functionally diverse group, sometimes with specialized roles, and 5) that phylogenetically different areas of the brain are involved in reward functions (in the case of mammals, several cortical and subcortical structures).

1.2. The complexity of reward functions

Reward functions are very complex in mammals and include various processes that Berridge for clarity divided into 'liking', 'wanting' and 'learning' components (Berridge, 2008, 2009; Berridge et al., 2009; Berridge and Kringelbach, 2015, 2013). 'Liking' is the main hedonic component of reward (pleasure itself), 'wanting' is the motivational process of incentive salience or the motivation to obtain the reward, 'learning' are classic and instrumental associations and cognitive representations of reward (Fig. 1). The 'wanting' process occurs at the beginning of the reward, the 'liking' more in the consumption phase, which leads to satiation, and 'learning' throughout the entire reward process.

The division of reward into these components led to a decrease in the dominant importance of the mesolimbic dopaminergic system in reward. This system plays a role in the 'wanting' process. Berridge assumes that the core of the 'wanting' process is realized mainly subcortically, in the brainstem structures where the basis of affects should be generated, mostly in the mesolimbic dopaminergic areas. The entire process of 'liking' is completed in the cortical parts (orbitofrontal cortex, prelimbic cortex and insular cortex), but the basis of affects (joy and pain) is based in the brainstem. Pleasure generation is concentrated into relatively small areas of the brain — the hedonic hotspots, which are situated cortically and subcortically (in addition, there are several places that minimize the feeling of 'liking,' called 'coldspots'). (Pecina and Berridge, 2005; Berridge, 2009; Berridge and Kringelbach, 2015). The functions 'liking' and 'wanting' are not centralized, but they are realized in different parts of the brain from the developmentally older brainstem regions to the younger cortical regions. Their function is hierarchized and synchronized. (Berridge, 2009). Given that both components of the reward are located cortically and subcortically (even a hedonic feeling occurs without cortical structures as subcortical areas are sufficient for hedonic reactions, Berridge and Kringelbach (2015)), it would be difficult to consider the hedonic function of reward, subjective pleasure, as an evolutionarily younger phase.

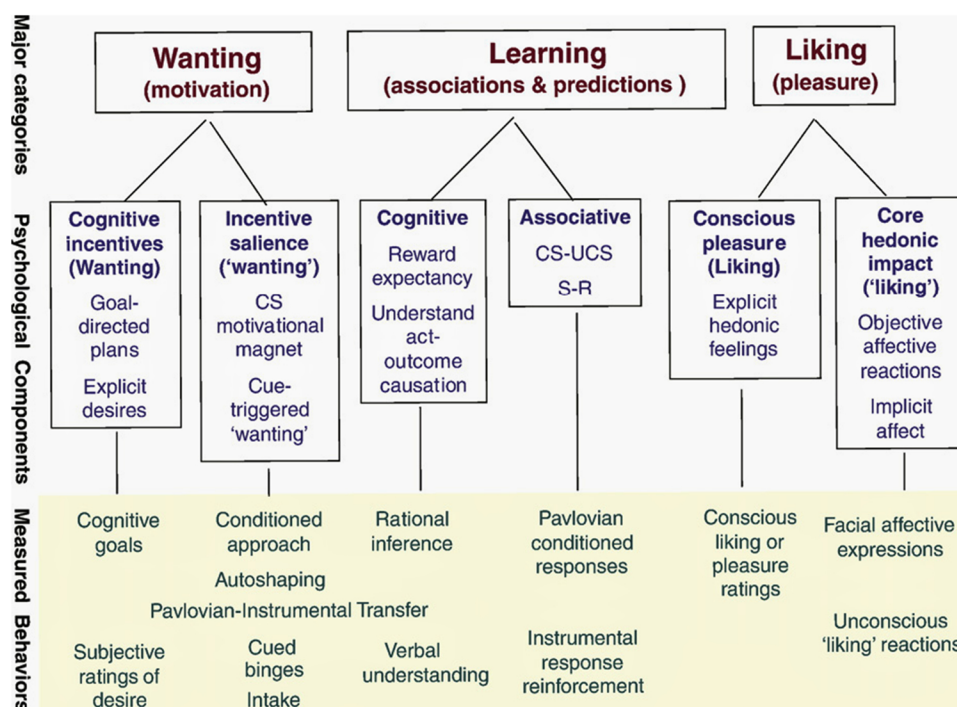


Fig. 1. Components of reward according to Berridge (2009). Reprinted with permission.

### 1.3. Insects and the reward system

Neuronal organizations, intracellular information cascades, neurochemical systems, and plasticity mechanisms are now viewed as evolutionarily conserved and occurring across the animal kingdom. However, the components of the reward system, as well as the mechanisms that increase an organism's likelihood of developing important survival behaviours, differ among various animal groups; for example, animals exhibit differences in the complexity of their brains or neurochemical equipment. The reward functions of mammals are associated with high quality brain networks, inter alia, because pleasure/displeasure is perceived as the basis of higher brain functions.

When studying these systems in insects, some basic questions arise concerning whether we can discuss their reward functions as features that are equivalent to the reward system of mammals. Do insects have neural substrate qualities that are sufficient for the hedonic component of reward — for the 'liking' component? And if the neuronal substrate has that quality, can we find in the insect brain neurochemical conditions similar to those known in the mammalian reward system (assuming the aforementioned universality of neurochemical systems and intracellular information pathways in animals), or are the reward functions built using different neurochemical systems?

The insect reward system has been investigated in several models (e.g. honey bee – Hourcade et al., 2010), however, the most extensively explored is the brain of the fruit fly *Drosophila melanogaster*, on which we will focus most of our discussion. It is important to realize that there are large evolutionary distances between the insect representatives, thus their brains differ significantly in complexity (e.g. Fahrbach, 2006). Naturally, there are differences in the organization of reward systems, reflecting the different life strategies of individual insect models (Mizunami and Matsumoto, 2017a).

*Drosophila* has a well-developed brain and a well-developed mushroom body (MB), a structure that has major roles in associative learning. At the same time, the *Drosophila* brain is simple enough to be studied in-depth. Considering the fact that its genome is completely described, gene manipulation techniques are well developed, breeding under laboratory conditions is easy, and the next generation of *Drosophila* can be acquired in ten days, *Drosophila* is an ideal laboratory model organism for the study of the brain as well as for other organ systems. Since the basic principles of the functioning and organization of the nerve cell and network are universal in the animal kingdom, knowledge of the brain of *Drosophila* can complement the knowledge of the human brain, and vice versa: the findings from mammalian/human brain research can inspire insect brain studies.

In this review, we describe the current state of view of the *Drosophila* reward system, with an emphasis on the possible presence of a hedonic component of reward, as well as the appropriate neural conditions for its presence. We hope to answer the questions (1) do *Drosophila* have a brain of sufficient quality for a fully developed reward system? (2) are there neurochemical systems in the *Drosophila* brain that are generally associated with the 'wanting' component? and finally, (3) are there neurochemical systems that would allow the function of 'liking', at least in the basic form? That is, is the discussion of hedonic function in *Drosophila* justified, or is this just an inadequate anthropomorphization? In describing the model of neuronal substrate, we emphasize the evolution of this view while highlighting the properties of neuronal background that complicate the creation of a simple model of *Drosophila*'s reward functions.

## 2. The complexity of neuronal substrates for associative learning and reward in *Drosophila*

### 2.1. Associative learning in *Drosophila*

We mentioned that for a long time, reward functions have only been studied in mammals — animals that undoubtedly have highly-developed

and complex brain. One of the main limitations of discussing the insect reward system is the uncertainty regarding whether such a relatively simple and short-living organism can have a brain substrate quality which would allow the complexity of reward functions. In the next section, we will try to describe the *Drosophila* brain network, including a discussion of its potential for a reward system.

The basic premise of the studying the reward system of *Drosophila* is that this fly can discriminate between rewarding and aversive environmental stimuli, and that the key to associative learning with memory formation is reward/punishment. *Drosophila* is known to seek reward and avoid punishment. *Drosophila*'s response to reward is often studied in context of behavioural manifestations or in the change in activity of particular network elements, but not in context of the full meaning of the term reward, including pleasure (see review Lowenstein and Velazquez-Ulloa, 2018).

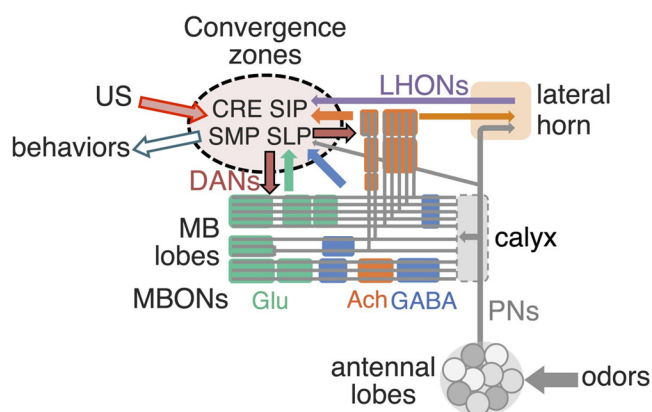
The process of associative learning is thoroughly described by the way olfactory information is processed and the association of this information with a positive unconditioned stimulus (US), most commonly sweet sugar, or a negative US, most commonly an electric shock or quinine. Nerve pathways and networks involved in olfactory associative learning with memory formation are described in detail and the entire connectome is rigorously identified (Fig. 2), (Aso et al., 2014a, 2014b; Kaun and Rothenfluh, 2017; Takemura et al., 2017). Also described are the intracellular structures and the process of information flow (an overview of intraneuronal mechanisms of associative learning in marine gastropod *Aplysia* with assumption of general validity in all animals: Hawkins and Byrne, 2016; Byrne and Hawkins, 2015; at *Drosophila* review Guven-Ozkan and Davis, 2014).

### 2.2. Differences in neuronal models of associative learning

Models of interconnections of key structures and views on the functions of individual elements of the reward system develop over time as our knowledge progresses, and the various models differ quite significantly from one another, ranging from a linear view to a computational view.

#### 2.2.1. Feed-forward models

The first models of the *Drosophila* reward system were feed-forward, describing specific connections between individual neurons in olfactory information processing, in search of reward principles focusing on the



**Fig. 2.** Schematic of MB lobes connections with target neuropil LH, CRE, SMP, SIP and SLP highlighted as convergence site according to Aso (2014a). The authors assume that this neuropil must receive information encoding an unconditioned stimulus such as sugar or shock for the DAN to obtain information for memory formation. In this model, the downstream circuits influencing behaviour have a source in the LH, CRE, SMP, SIP, and SLP. LH - lateral horn, SLP - superior intermediate protocerebrum, SIP - superior intermediate protocerebrum, SMP - superior medial protocerebrum, CRE - crepine neuropil. Reprinted with permission.

sites of convergence of unconditioned stimulus (appetitive/aversive stimulus) with conditioned (CS) stimulus (initially neutral stimulus, typically odour). The classical neuronal line of this model is based on four synapses: 1. a synapse between an olfactory sensory neuron and a projection neuron in the antennal lobe glomerulus, 2. a synapse between axons of projection neuron and dendrites of Kenyon cells in the calyx of mushroom body, 3. a synapse between Kenyon cell axons and MBON in the mushroom body lobes. The reward value in this model was represented by the 4th synapse — a connection of DAN axon to the presynaptic part of the KC > MBON synapse. In this original concept, the point of convergence of the neutral CS (odour) coming through the KC path and the reinforcement US signal coming through the DAN path should be at the KC > MBON synapse. Simultaneous activation of CS and US representations at KC with subsequent MBON activation is a condition for synaptic changes as a basis for associative learning and the site where the association of environmental stimulus with reward is expected to occur (Schwaerzel et al., 2003; Riemensperger et al., 2005; Busto et al., 2010; Waddell, 2010; Perry and Barron, 2013). Nevertheless, other sites of possible convergence are also described: in the antennal lobe or the MB calyx of *Drosophila* (Yu et al., 2004; Thum et al., 2007; Ueno et al., 2013) or on the target place of MBON output, including the crepine (CRE), superior medial protocerebrum (SMP), superior intermediate protocerebrum (SIP), superior lateral protocerebrum (SLP) and lateral horn (LH) (Aso et al., 2014b).

2.2.2. Feedback and recurrent circuits

The emergence of other models began to disrupt the idea of linearity by introducing the idea of feedback loops (Figs. 3 and 4), or more precisely, recurrent circuits, and emphasizing the importance of other cell types, mainly the anterior paired lateral neuron (APL) and dorsal paired medial neuron (DPM) (Aso et al., 2014a; Aso et al. al., 2014b; Ichinose et al., 2015; Aso and Rubin, 2016; Felsenberg et al., 2017; Oswald and Waddell, 2015; Cognini et al., 2018). This is a significant change not only in that it implicated the association of other synapses in the neural pathway, but more importantly because it represents the shift in viewing the flow of neural information as a feed-forward phenomenon to that of reverberating circuits, in which activity can be maintained for longer periods with no additional sensory input, without a clear distinction of output from input. The existence of recurrent circuits with the possibility of reverberating activity has been documented at all levels of the neural pathway for the formation of olfactory association with reward/punishment: e.g. between DAN and MBON within the same MB compartments and between compartments or among MBON, DAN and

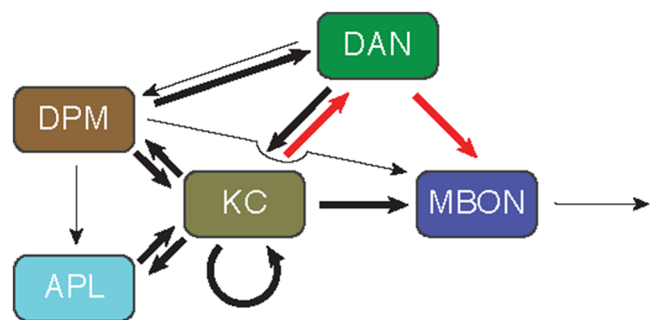


Fig. 4. Schematic of primary connectivity motifs in MB according to Takemura et al. (2017). Thick arrows = connections composed of >200 synapses, thin arrows >50 synapses in at least two compartments, and connections with fewer than 50 synapses are not represented in this schematic picture. Reprinted with permission.

MBON target neurons (Aso et al., 2014a; Ichinose et al., 2015), between MP1 DAN and a pair of GABAergic MVP2s (= MBON-gamma1pede) (Perisse et al., 2016). Recurrent and hierarchical connectivity between MBON and DAN allows re-evaluation of memory based on a reward prediction error. The aversive DANs overlap the MBON dendrites that drive the behavioural approach, while the rewarding DANs overlap the avoidance dendrites (Aso et al., 2014b).

Recurrent circuits exist also between KC and DAN; KC synapses onto DAN, more than DAN onto KC, contrary to the original idea of feed-forward information flow in association forming (Takemura et al., 2017). A local recurrent microcircuit, likely composed of output synapses from KC dendrites, was later reportedly found in the MB (Zheng et al., 2018).

Important feedback and recurrent circuits are formed by the APL and DPM neurons, which are intrinsic to the mushroom body and branch out across all lobes and calyces of the MB to create reversible circuits, especially with the KC. APL is GABAergic and mainly functions in the negative feedback necessary to maintain sparse coding (Lei et al., 2013; Lin et al., 2014). DPM has a major role in memory consolidation and sleep regulation (Keene et al., 2004; Yu et al., 2004; Keene and Waddell, 2005; Keene et al., 2006; Krashes et al., 2007; Krashes and Waddell, 2008; Pitman et al., 2011; Cervantes-Sandoval and Davis, 2012). There are numerous gap junctions between APL and DPM neurons, enabling the balance between the excitatory activity of DPM with the inhibitory activity of APL (Pitman et al., 2011; Wu et al., 2011). Furthermore, a feedback loop between DPM and KC has also been described (Pitman et al., 2011; Wu et al., 2011).

2.2.3. Network of cognitive functions and reward

Attempts to understand the functioning of MB (and any other brain network) were largely influenced by the concept of the classical reflex arc, wherein sensory information reach the cell (centre) in and proceed to the effector neuron (organ) in an organized manner.

Imagining the association between two parallel inputs is complicated, let alone tens or hundreds of inputs. *Drosophila*'s mushroom body and its input and output was later described in detail (e.g. Aso et al., 2014a, b; Cohn et al., 2015; Takemura et al., 2017; Zheng et al., 2018) and it turned out that even in the short-living fly, the MB forms a very complex network. The idea of feed-forward and well-arranged connections was complicated by the number of cells involved and the expected number of synapses. As a rough estimate, the MB network consists of approximately 2200 KC of 7 types, 34 MBON of 21 types (cholinergic, glutamatergic, GABAergic), and 130 DAN of 20 types (clusters PAM and PPL1). Different MBONs are modulated by different DANs, MBON dendrites are located in 15 compartments, three types of MBON and one type of DAN interconnect in multiple compartments, and MBON axons project to five distinct neuropils — crepine, superior medial protocerebrum, superior intermediate protocerebrum, superior lateral protocerebrum and lateral horn —

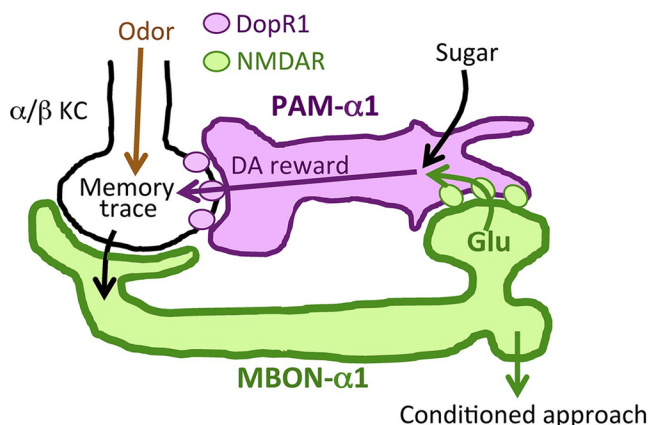


Fig. 3. A simple feedback circuit model according to Ichinose (2015). The dopamine effect (here from PAM-α1) on the associative process is associated with sugar reward and LTM formation is influenced by glutamatergic feedback from MBON-α1. Abbreviations: Glu - glutamate, DA - dopamine, DopR1 - dopamine receptor, LTM - long-term memory, NMDAR - glutamate N-methyl-D-aspartate receptor. Reprinted with permission.

in which 90 % of DAN dendrites are found. Some MBON axons project to DAN dendrites in the same compartment. Each MBON receives inputs from 90 to 2000 KC from 1 to 2 compartments, and each KC forms a synapse with 5–6 types of MBON (Aso et al., 2014a).

#### 2.2.4. Computational MB modelling

Computational modelling of these networks has furthered the understanding of the function of neural networks involved in olfactory learning, and has clarified the role of network science in mapping the structure and function of the brain (Deco and Kringelbach, 2014). These models attempt to describe the sequence of olfactory information processing and the association process in the MB using mathematical language. Computational models of these relatively simple insect brain networks may also potentially be adapted to examine decision-making functions of the brain with changing environmental conditions in robotics. However, they first and foremost provide important information regarding the insect brain itself. Firstly, the tests performed with the MB computational model provide information about what elements and functionalities of the network are essential for learning. Secondly, they use concepts from the whole network science to create theoretical frameworks of MB activities, which are concepts that may be extremely difficult to describe when solely depending on biological sciences to analyse the individual network elements independently (overview of basic elements for the architecture of neuro-inspired computational models Patanè et al., 2018a).

The first computational models were based on a modular way of organizing the brain and captured the aforementioned main neuronal connections of the olfactory system as well as some feedback relationships, but excluded many complicated elements (Huerta et al., 2004; Nowotny et al., 2005; Wessnitzer and Webb, 2006; Smith et al., 2008; Arena et al., 2010; review of computational olfaction models of *Drosophila* Mosquero and Huerta, 2014; Gupta et al., 2018).

The basic architecture of these models is similar. A three-layered network (AL layer, intrinsic KC layer, MB lobes layer) is involved in the olfactory learning process. Other networks that play a key role is LH (regularly inhibits the KC layer and performs a reset after the last signal detection, Smith, 2008) and source of reward values (Huerta and Nowotny, 2009). The olfactory network can thus be modelled as a single-hidden layer neural network with one hidden layer of KC (Huerta et al., 2004; Huerta and Nowotny, 2009; Montero et al., 2015). The basic process consists of the nonlinear transformation of signal from AL to KC using non-specific connectivity, sparse data encoding in KC, linear classification of KC activity patterns by MBON (Huerta et al., 2004; Nowotny et al., 2005), followed by Hebbian learning at synapses: Miconi (2015) or Delahunt et al. (2018) describe the modulation of Hebbian learning by reward.

Indeed, computational models ignore the asymmetries and irregularities of the biological pattern and necessarily remain simplified. The modular architecture of the network is based on the idea that the brain is divided into smaller modules (relatively separate networks) where each module performs a specific function and the individual modules more or less compete with each other. Network modularity almost certainly cannot explain certain network features (Kim and Kaiser, 2014). Arena et al. (2016) and Patanè et al. (2018b) described the new MB architecture in accordance with the neural reuse theory (Anderson, 2010: Reuse of neural circuits for various brain functions is a common organizational principle). Individual networks do not work modularly. They are not designed for one particular function, but the same neuronal circuits may be used for different functions as needed. For example, the MB may have simultaneous functions in decision making (classification), motor learning, sequence learning, attention, and expectation. (There may also be a new perspective regarding the controversial role of dopamine (DA) in various functions - according to the neural reuse theory, the same circuitry may have a role in different neural functions).

The development of computational models of olfactory insect learning is directed towards dynamic, holistic and stochastic models,

which work with uncertainty. In 2018, Dalgaty et al. (2018) applied the concept of a liquid state machine in the computational model of MB. Similarly, Arena et al. (2015) modelled the MB architecture in an attempt to make it more dynamic on the liquid state network. Mosquero and Huerta (2014) recall the stochastic essentiality of sensory perceptions and even of learning rules. Nowotny et al. (2005), in describing the olfactory data classification in the computational model of MB *Drosophila*, states: 'We are able to show that there is no need for an explicitly or algorithmically specified connectivity. The classification works on a purely statistical basis'.

#### 2.3. The network is further complicated

The result of MB research therefore reveals a very complicated network. Though considerably less complex than mammalian brain networks, it is still too complex to be described using a feed-forward model. It is evident that the description of the MB functioning must be based on the characteristics of the whole network, not on the individual network elements. Research of the human brain has fallen into a similar trap — the ongoing detailed description of the network elements does not lead to the understanding of the whole brain (this is incidentally one of the reasons why it is attractive to study *Drosophila* in neuroscience). Evidence of the recognized complexity of the *Drosophila* brain may be responsible for the shift from studying the adult brain to the brain of the early larval stages of *Drosophila* (e.g. Honjo and Furukubo-Tokunaga, 2009; Selcho et al., 2009; Eichler et al., 2017).

The neural background of association learning is not always as simple, regular and defined as it is in our models. On the contrary, they are complicated by various factors that prevent clear modelling and understanding of the organization of neuronal bases and their functioning. Here are some of the elements that complicate the understanding of association learning:

- 1) Relative autonomy of the individual MB compartments: Formation and consolidation of association links (memory) occur independently and in parallel in individual MB compartments, different learning rules are applied in parallel, and the connectivity of each cell develops autonomously (Aso and Rubin, 2016; Takemura et al., 2017).
- 2) Irregularity in network architecture: Despite the complexity and the relatively high number of MB network elements, there are places where one neuron decides on a particular function. For example, 14 MBON types contain only one cell per hemisphere (Aso et al., 2014a). The organization of connectivity is different in each MB lobe (Aso et al., 2014a; Takemura et al., 2017).
- 3) In many MB compartments, no specialisation is described, although other parts of the MB network are studied for precise, specific effects, such as participating in a particular type of memory or in a specific form of learning (e.g. Keene et al., 2006; Yu et al., 2006; Krashes et al., 2007; Krashes and Waddell, 2008).
- 4) Co-transmission: it appears that the classical understanding of relationships through the effects of particular neurotransmitters/modulators is not applicable other than for educational purposes. A large proportion of cells in the MB, including key DAN structures, are multi-transmitters. Understanding of the effects of two transmitters from one cell remains to be elucidated (Takemura et al., 2017; Croset et al., 2018; Nässel, 2018). Contrary to current beliefs, it is not only a co-release of the major neurotransmitters (small molecule neurotransmitters) with neuropeptides, that modulate the output of the main transmitter without changing the main transmitter substantiality, but also the co-release of several major neurotransmitters (an overview of the co-transmission of neuropeptides and small molecule neurotransmitters is provided by Nässel (2018) and Croset et al. (2018)) or cotransmissions of nitric oxide (its role as a cotransmitter in some DAN subsets and its importance for different memory dynamics described Aso et al. (2019)).

- 5) Indistinct role of non-metabotropic junctions: Traditional understanding of neuronal function is mostly focused on the flow of information through metabotropic connections, and the interpretation of the role of non-metabotropic connections of two neurons (ionotropic or gap junction) remains unclear. In the context of the MB, gap junctions are present between KC to potentially enable lateral signal propagation (Takemura et al., 2017), as well as between APL and DPM neurons (Wu et al., 2011).
- 6) Non-linear signal flow: The signal does not have to flow only from the presynaptic area to the postsynaptic area (linearly). Except for the aforementioned axonal gap junction connection, the description of the mixture of synaptic output and input of the KC dendrites and the MB-CP2 cells (neurites are pre- and post-synaptic concurrently) indicates the possibility of nonlinear information flow (Zheng, et al., 2018). Similarly, the idea of targeted linearity is disturbed by the simple fact that dopamine (the key element in the reward system) extends extracellularly over a certain distance outside the synaptic cleft (Ueno and Kume, 2014: non-dopaminergic cells in experiment with an artificially expressed dDAT uptake DA from the synaptic space). The information flow in associative learning and memory formation can generally be bidirectional — diffusible retrograde messengers (e.g., nitric oxide), which are released after spiking activity from the post-synaptic neuron (MBON) back to the pre-synaptic neuron (KC) and are involved in the formation of plasticity, are likely to play an important role in memory formation and association learning (The significance of associative learning in *Drosophila* was demonstrated via computational models by Smith, 2008 and Faghihi et al., 2016).
- 7) Rhythmic oscillation and synchronized activity of the network components: The functional significance of one-time neuron activity is expected to differ from that of rhythmic oscillation of neuron activity (Rosay et al., 2001; Placais et al., 2012; Placais and Preat, 2013). Moreover, there is yet another significance in the synchronization of the activity among the various components of the network (Tanaka et al., 2009). The oscillation of activity may be caused by signal re-entry circulation, or may be the result of the interplay between inhibition and excitation (e.g., GABAergic and cholinergic signal in KC, Rosay et al., 2001). Activity oscillations (spontaneous calcium oscillations in MB-MP1 and MV1 neurons) have been described as satiety sensitive — they occurred after the administration of energy-valuable sugar, which reflects a state of satiety (Musso et al., 2015; Placais and Preat, 2013). Yet another significance may be the periodicity of the oscillations, although it does not appear to be as typical of *Drosophila* as in locusts (Turner et al., 2008).
- 8) Uncertainty of random activity patterns: Even in *Drosophila*, odour is combinatorically represented in the MB, in the form of sparse activity patterns of a randomly assembled set of KC (e.g. Turner et al., 2008; Campbell et al., 2013). Activity patterns of close odours may overlap and the fly may respond to the new odour in a manner associated with the previously learned odour (Campbell et al., 2013). Activity pattern overlap allows the prediction of response to new, similar odour; this process may be understood as a generalization of a previously known association to a new odour after association learning or as an adaptation to a new odour without learning. The difficulty in understanding the reward function arising from the nature of sparse activity can be summarized into a simple question: if sparse activity is random, how can DAN mark a randomly selected set of active KCs with reward value? Sparse coding is likely to be a universal network strategy (Olshausen and Field, 2004).

#### 2.4. Lateral horn, not mushroom body?

An important brain structure for controlling behaviour in response to an external stimulus (odour in the case of *Drosophila*) is the lateral horn (lateral protocerebrum). In the classic conception, it is not known to

have a role in associative learning or reward functions, but rather in controlling innate behaviour in the form of innate responses to a strong odour — some of the axons of PN in the AL go to the MB for associative learning while others project to the LH for efficient connection to elicit the effector motor response as a dedicated neural channel, in accordance with the labelled line principle (Heimbeck et al., 2001; Busto et al., 2010; Scaplen and Kaun, 2016).

But the role of the LH may be more prominent in associative learning. Huoviala et al. (2018) described the neuronal pathway of a strong aversive reaction via the LH as much more complicated than the simple notion of labelled line (e.g., with axo-axonal connections of PN axons to the LH, with great convergence and divergence of signal in the LH). Shih et al. (2015) described the connections between the AL and LH as much stronger than those between the AL and MB. A model outlining an important role of LH in *Drosophila*'s associative learning has been described by Galizia (2014). According to them, the odour evaluator is not the MB (it performs odour identification, which is reinforced by associative learning), but instead the LH, where reward value appears to be assigned. All PNs that project into the MB and also project into the LH are mostly excitatory and uniglomerular. Multiglomerular PNs go directly to the LH and are inhibitory. The valence assigned to the odour in the LH varies based on context (which is apparently determined by the neuropeptides that are present). The MB influences the LH by controlling its inhibitory output to the LH. The inhibitory output of the MB in response to odour decreases when the odour has been subject to associative learning; thus, learning leads to the MB disinhibition of the LH evaluator system (Fig. 5).

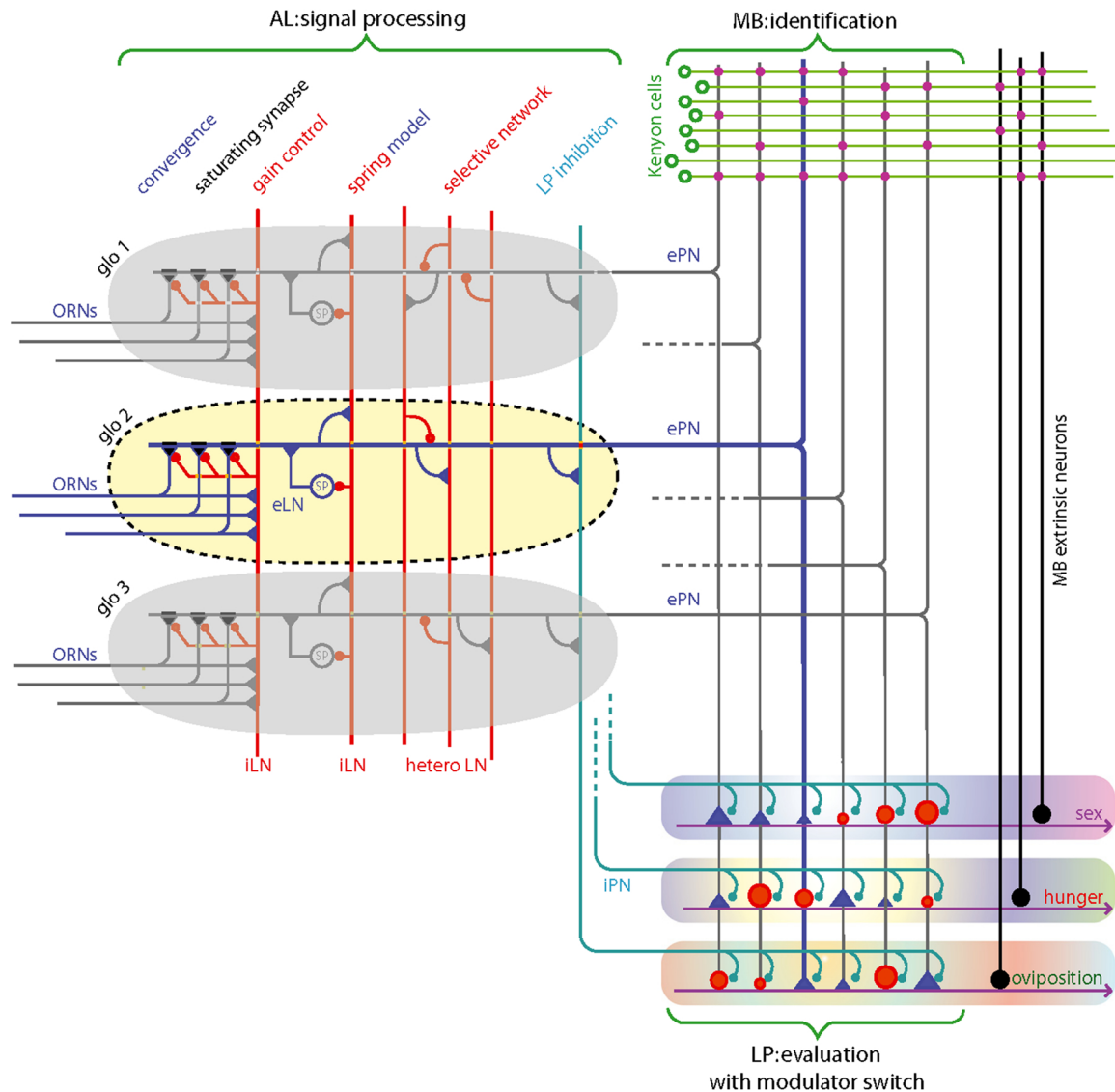
A basic model of one of the possible roles of LH (the integration of innate olfactory input from PN and learned sensory information from MB) was proposed by Dolan et al. (2018). They identified two excitatory LH output neuronal cell types (PD2a1 and PD2b1) postsynaptic to MBON- $\alpha$ 2sc, confirmed their direct uniglomerular PN input mostly from appetitive olfactory channels, and demonstrated their dual behavioural role. These neurons are necessary for unlearned attraction to some odours and also during aversive memory recall. The authors described a circuit modulating the approach behaviour (reduced attractive drive) due to aversive memory retrieval.

An interesting insight into the role of the LH in the associative olfactory learning process of insects is offered by the computational models of this process (Huerta et al., 2004; Nowotny et al., 2005; Smith et al., 2008). In these models, inhibitory activity from the LH increases the sparseness of KC activity and resets KC activity upon prior detection of the signal. Computational models, however, do not address how the LH directly processes olfactory stimulus.

### 3. Neurochemical systems for the 'wanting' component in *Drosophila* — the role of dopamine

#### 3.1. The role of dopamine in the reward system of *Drosophila*

It has already been mentioned in the previous section that dopaminergic neurons (and OANs) play a significant role in associative learning and represent, as in mammals, a key neurochemical system for reward functions. However, if we assume truth of the aforementioned concept of the MB as a complex neural network with many elements, synapses, co-releases of neurotransmitters/neuromodulators and asymmetric architecture, it is difficult to accept that one of the network elements alone (dopaminergic neuron in our case) and one of its neurotransmitters/neuromodulators is what stimulates the reward system. When explaining reward functions, the word dopamine is often used as an unexplained but also an all-explanatory symbol, imparting a simple statement that dopamine carries information that reflects reward value. Investigation of the reward system was initially based on the key role of biogenic amines in controlling reward response in mammals (Ikemoto et al., 1997; Schultz, 1997; Spanagel and Weiss, 1999). Naturally, the biogenic amines drew interest as potential candidate substances involved in modulating



**Fig. 5.** Representation of the insect olfactory system illustrating the role of the lateral horn in the process of forming associations, according to Galizia (2014). Signal processing in AL, odour identification in MB, odour evaluation in LP. Abbreviations: ORN - olfactory receptor neuron, ePN - excitatory projection neuron, iLN - inhibitory interglomerular neuron, eLN - spontaneously active excitatory interglomerular neuron, LP - lateral protocerebrum. Excitatory connections are symbolised by blue triangles, inhibitory connections by red circles. Reprinted with permission.

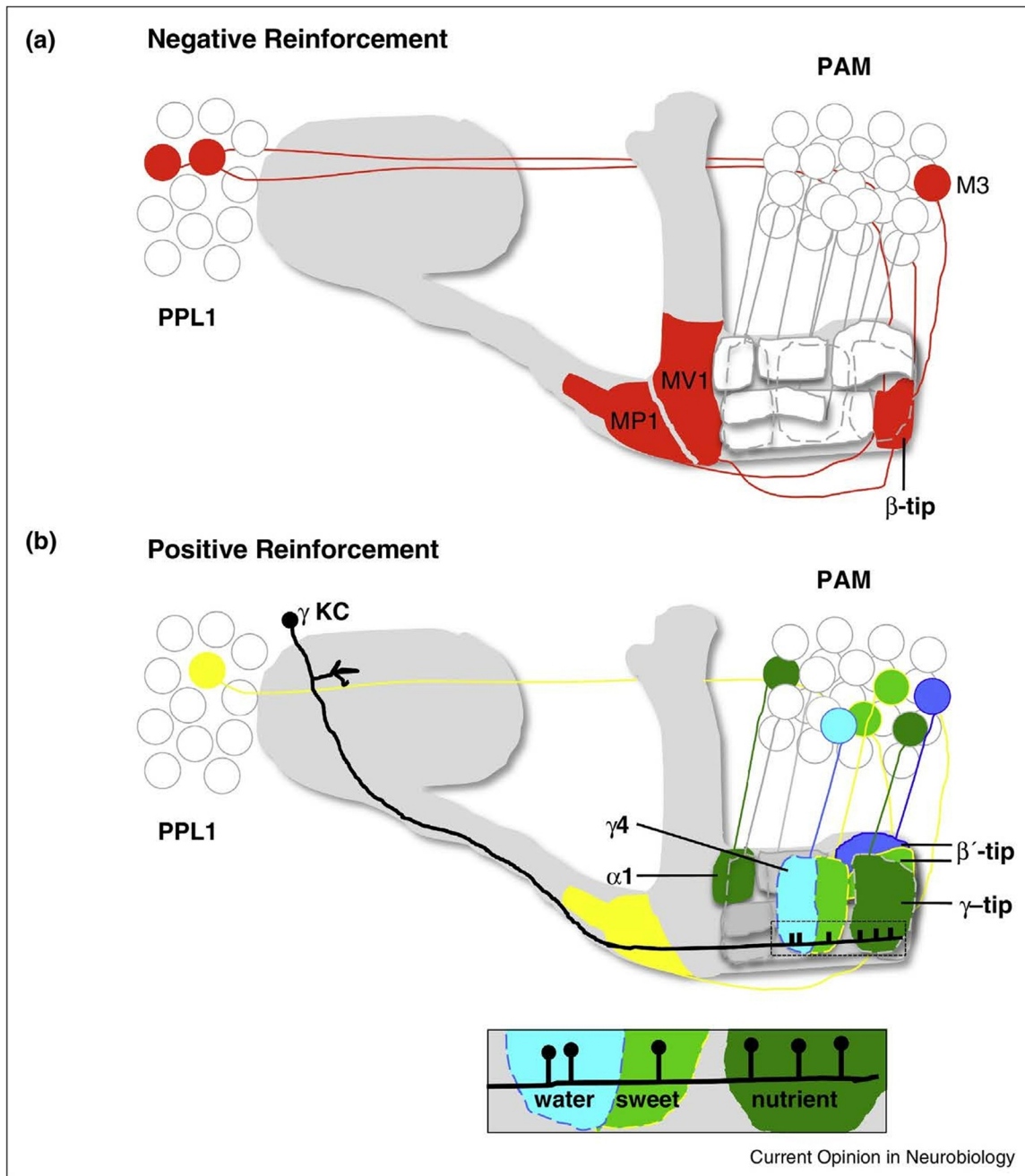
associative processes and reward in *Drosophila* and other insects. What is the role of biogenic amines in reinforcement and reward response in *Drosophila*? What is the real bearer/signal/agent of the reward?

The initial concept of reward mediation in insects was that dopamine carries negative reward value information and causes aversive learning and avoidance behaviour. Positive reward value, appetitive learning and approach behaviour is mediated by octopamine (Schwaerzel et al., 2003; Riemensperger et al., 2005; Vergoz et al., 2007; Busto et al., 2010). Dopamine was subsequently confirmed to have a role in appetitive learning as well (Kim et al., 2007; Liu et al., 2012; Burke et al., 2012). The role of OA has gradually diminished, with its importance only remaining in forming short term memory (STM) for sugar sweetness, but not for nutritional value (Waddell, 2010; Burke et al., 2012; Huetteroth et al., 2015).

The dichotomous roles of amines (octopamine as a key element for appetitive learning, dopamine for aversive learning) remain in other insects such as in the bee *Apis mellifera* (Faroquii et al., 2003; Vergoz et al., 2007; Tedjakumala and Giurfa, 2013; Tedjakumala et al., 2017) or in the cricket *Gryllus bimaculatus* (Mizunami et al., 2009, 2015). Reinforcing mechanisms are apparently not uniform in insects. The role of

dopamine in aversive learning appears to be conservative in insects and mammals, but the role of amines in appetitive conditioning is ever-changing (Mizunami and Matsumoto, 2017a).

The *Drosophila* brain is supplied by dopamine from many types of DAN. Two DAN clusters — protocerebral anterior medial (PAM) and protocerebral posterior lateral 1 (PPL1) — project into the MB lobes (Mao and Davis, 2009; Aso et al., 2014a; Aso and Rubin, 2016). There are 130 DAN of 20 types; different MBONs receive the same input from KC, but are modulated by different DANs (Aso et al., 2014a). The majority of PAM neurons are involved in positive reinforcement (Mao and Davis, 2009; Aso et al., 2012; Burke et al., 2012; Liu et al., 2012; Ichinose et al., 2015; Huetteroth et al., 2015; Yamagata et al., 2015; Perisse et al., 2016; Oswald and Waddell, 2015), the majority of PPL1 cluster neurons are involved in negative reinforcement and aversive learning (Claridge-Chang et al., 2009; Aso et al., 2012; Oswald and Waddell, 2015) (Fig. 6). However, PAM and PPL1 neurons are functionally heterogeneous, some PAM cluster neurons are involved in aversive learning (Aso et al., 2010) describe the role of M3 neurons in aversive learning, Yamagata et al. (2016), the role of PAM-Y3 neurons in aversive and appetitive learning, some PPL1 neurons participate in appetitive



**Fig. 6.** The specialisation of main DAN clusters in positive and negative reinforcement according to [Owald and Waddell \(2015\)](#). a) Negative reinforcement: MB-MP1 [PPL1-γ1pedc] and MB-MV1 [PPL1-γ2α'1] DANs from the protocerebral posterior lateral 1 cluster, MB-M3 (PAM-β2β'2a) neuron from the protocerebral anterior medial cluster. b) Positive reinforcement: DANs in the PAM cluster. Reprinted with permission.

learning in addition to aversive learning (the role of the MP1 neuron in memory consolidation in aversive learning is described by [Placais et al. \(2012\)](#) and [Aso et al. \(2010\)](#), the role of the same neuron in forming appetitive LTM is described by [Musso et al. \(2015\)](#)).

Dopamine cells do not mediate a particular motivational value independently of the surrounding network. What value dopamine modulation attributes to an odour depends on where (in which MB lobes) the KC > MBON synapse is modulated and which MBONs are involved in the synapse. The basic valuation system for attribution of value, according to [Aso et al. \(2014b\)](#), is likely the neuronal interconnection of 15 MB compartments. Behavioural valence correlated with MBON neurotransmitter type: all MBON eliciting aversion were

glutamatergic, all MBON eliciting attraction were cholinergic or GABAergic.

The recent work of [Handler et al. \(2019\)](#) however illustrates that both PAM and PPL clusters are sufficient to produce appetitive or aversive associations depending on whether the odour precedes or follows the reinforcement in time. Accordingly, valence should be caused more by the order and timing of an associated stimulus than by a particular type of DAN. Further, the authors demonstrated a rapid change in valence by changing the timing and order of the stimulus.

Another form of bidirectional valence coding in the same DAN is described by [Yamagata et al. \(2016\)](#). The authors describe the participation of PAM-γ3 neurons in appetitive and aversive reinforcement due



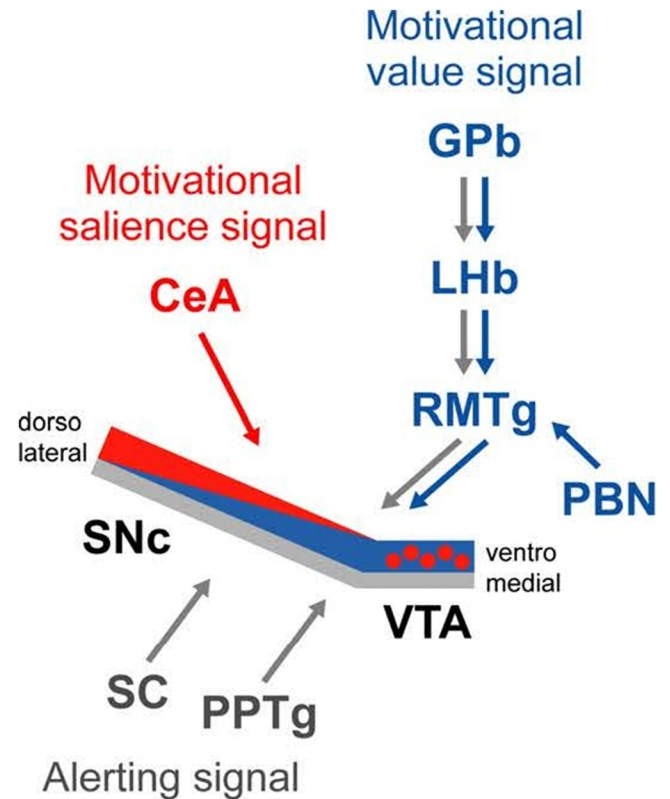
to activity change. The activity of these neurons mediates aversive memory, and transient inactivation of their basal activity induces appetitive memory formation. Hence, excitation and inhibition of DAN may mediate appetitive and aversive value. As mentioned below, PAM-Y3 neurons are a target of the possible satiety-signalling neuro-peptide allatostatin A.

### 3.2. The role of dopamine in the reward pathway of mammals, humans and animals in general

But what information does dopamine carry? Learning the significance (pleasure value) of a new stimuli can theoretically take place by 1) direct pleasure marking, 2) evaluating the result of the response to a new stimulus, 3) comparison with already known stimuli (innate or learned) or 4) linking to an overall generalized pleasant state. Thus, the US should be associated with a reward, a mechanism comparing a new one to a known one (prediction error), or with a mechanism carrying information about the overall calm, filled state of the animal (satisfaction). These comparisons can probably happen with varying degrees of difficulty at all levels of the connectome for sensory information processing — from the baseline level of the first neurons (sensory neurons) to the complicated level of the higher associative centres. There are major hypotheses about the role of DA in the reward process of mammals (and humans), such as the one proposed by Berridge (2008). The most powerful ones are the hedonia hypothesis, where DA is a pleasure neurotransmitter and a bearer of subjective feelings of liking and pleasure, reward learning hypothesis, where DA is a teaching signal or prediction generator, and incentive salience hypothesis, where DA is a tool for focusing attention and labelling motivational significance.

Similarly, in the case of *Drosophila*, the original hypothesis was that dopamine is a hedonic neurotransmitter (however, the assumed hedonic 'liking' component is absent, see below). Assessment of the importance of dopamine in reinforcing functions has shifted towards the reward prediction error theory, i.e. the role of dopamine in the 'learning' component (mammalian brain prediction error theory e.g. Rescorla and Wagner, 1972; Schultz, 1997; Bissonette et al., 2014) and the 'wanting' component (Berridge, 2009; Berridge and Kringelbach, 2013), or the non-rewarding dopamine alert/arousal hypothesis, explained below. Prediction error theory in insects is rarely studied; in crickets, it was used to explain a group of experiments (Terao et al., 2014; Mizunami et al., 2017b), in *Drosophila* it was used sporadically (Felsenberg et al., 2017), or it was mentioned as an alternative interpretation of results (Kaun et al., 2011). In a series of experiments focused on more complex forms of learning in *Drosophila*, Young et al. (2011) did not find the phenomenon of 'blocking': a stimulus previously paired with an US could block subsequent association of a second stimulus to the same US when the two stimuli occur simultaneously. The 'blocking' is due to the requirement of surprise for learning and is understood as a possible manifestation of prediction-error ability.

In the case of mammalian brain studies, the controversy between the hedonic hypothesis and prediction error theory has moved closer to integration: in the mammalian brain, all hypotheses about how dopamine functions in reward/reinforcement functions are likely to be concurrent. There are several intrinsically differentiated types of DA neurons in the mammalian brain with different types of interconnections and roles in motivation processes: some groups of DA neurons respond to the reward itself; some to the reward prediction; some to both; and some to non-reward function, such as surprising, novel, salient, and aversive stimuli (Schultz, 2006; Blomberg-Martin et al., 2010; Cohen et al., 2012). There are concurrently DA cell populations that are inhibited by aversion and excited by reward (encode the motivational value in the form of a prediction error signal), others respond equally to aversion and reward (encode motivational salience, corresponding simultaneously to arousal theory) (Blomberg-Martin et al., 2010; Schulz, 2006) (Fig. 7).



**Fig. 7.** Multiple function of DA neurons in different circuits according to Blomberg-Martin (2010). The role of DA neurons in motivational value, salience, and alerting signals depends on the signal source: motivational salience signal comes from central amygdala (CeA). Motivational value and alerting signals come from the pallidus border globus (GPb), lateral habenula (LHb), and rostromedial tegmental nucleus (RMTg). Aversive value signals come from the parabrachial nucleus (PBN). Alerting signals also come from the superior colliculus (SC) and pedunculopontine tegmental nucleus (PPTg). Reprinted with permission.

### 3.3. Dopamine and synaptic weight change

Regardless of whether dopamine/octopamine carries information about reward valency or error prediction, it is demonstrated that the primary mechanism of its influence is by modulation of the KC > MBON synapse. In association experiments, it is presupposed that the co-activation of CS and US representations on Kenyon cells with activation of specific output neurons is a prerequisite for changes in synaptic connectivity. In the classic model, the US representation is constituted by specific DAN groups, which change the synaptic weight of KC > MBON connection with their phasic bursts; these phasic bursts signal a deviation between expected and actual reward, an equivalent to coding the prediction error (Schwaerzel et al., 2003; Claridge-Chang et al., 2009; Aso et al., 2010; Waddell, 2010; Liu et al., 2012; Burke et al., 2012; Aso et al., 2012; Huetteroth et al., 2015; Ichinose et al., 2015; Oswald and Waddell, 2015; Yamagata et al., 2015; Cognini et al., 2018). There is a relatively small number of MBON in *Drosophila* MB — only 34 cells of 21 types in each hemisphere (one significance of this fact is a high signal convergence rate at the KC > MBON synapse, with approximately 2000 KC connected to 34 MBON) (Aso et al., 2014a).

Interpretative complications arise with the finding that the connection of DAN to both KC and MBON is more complicated than its representation in a simple model (Fig. 4). According to Takemura et al. (2017) only 6% of KC > MBON synapses have direct projections from DAN. The primary DAN targets are KCs, but DAN output also project onto MBON, APL and DPM. KCs synapse onto DAN even more frequently than DAN > KC, and not all KCs have inputs from DAN.

Reward information is a result of combinatorial pattern of excitation and inhibition of multiple DANs in different compartments (DAN modulates KC > MBON synapse in both directions, depresses and strengthens) rather than the result of the action of individual DANs. DANs act in an interconnected network to code information from the external context and the internal state of organism and direct olfactory signal to different postsynaptic targets — the signal is sent to different patterns of output activity (Cohn et al., 2015). The modulation of these synapses is not the same in all compartments. For example, in the alpha2 compartment, all KC > MBON synapses have inputs from the same DAN and they are modulated in a coordinated way; however, in the alpha1 compartment, they are modulated differentially. Obviously, there are several patterns and rules of architecture and modulation of synapses that exist in parallel (Takemura et al., 2017; Hige et al., 2015).

Modulation by the DAN does not seem to have an absolute valence. The same DAN signal can affect plasticity on the KC-MBON synapse bidirectionally (and can lead to an act of approach or avoidance), depending on whether DAN activity follows or precedes the neutral stimulus. In this bidirectional plasticity, DopR1 and DopR2 and consequently different intracellular signalling pathways play opposing roles and work very flexibly in balance (Handler et al., 2019).

### 3.4. Uncertainties about the role of DA remain

Let us adopt a simple model that DAN alters synaptic weights in the KC > MBON synapse and that this is the location of associative learning and memory. That is, DAN brings information about reward and punishment to the system. Two questions remain:

- 1) Are we really talking about reward and punishment? Research of mammalian brains and the postulation of the role of the DA system as a reinforcer are older and they certainly influenced the interpretation of the role of DA in insect brains. But is it the unpleasant that the *Drosophila* avoid? And is what the *Drosophila* seek indeed pleasant?
- 2) How does DAN detect reward and punishment? The idea of simple convergence of the US and CS signal is disrupted not only by the more complex interconnection architecture, but also by the question of how in reinforcement process DANs obtain information about the US or the external context. Many studies describe the role of DA and DAN in the reinforcement process of *Drosophila*, but the input of DAN is not described much, except the information on intrinsic metabolic satiety (e.g., Krashes et al., 2009; Root et al., 2011; Wang et al., 2013; Branch and Shen, 2017).

It is assumed that the DAN input comes from many areas of the brain (Mao and Davis, 2009; Aso et al., 2014a). It is also confirmed that DAN directly affects MBON activity without sensory input (Cohn et al., 2015; Takemura et al., 2017). However, DANs are part of several recurrent circuits, thus the expected information about external context and internal state (including eventual US, regardless of whether it is information about reward value or prediction error) can theoretically be introduced into the DA system from any level of the recurrent loop. For example, KC-MBON-DAN-KC and MBON-DAN-MBON have been described (Aso et al., 2014a; Ichinose et al., 2015; Perisse et al., 2016; Felsenberg et al., 2017; Cognigni et al., 2018). An interesting circuit is described by Aso et al. (2014a): DAN-MBON-target neuropil-DAN (target neuropil here is crepine, superior medial protocerebrum, superior intermediate protocerebrum, superior lateral protocerebrum, lateral horn). They assume that this target neuropil could receive US (reward, punishment) information (Fig. 1). Recall the report by Schultz (2006), which stated that DAN is oriented by the resulting behavioural response rather than by sensory input. DAN signal amplification/attenuation can also occur without any external information, only based on the characteristics and activity of the network itself. Similarly, in mammals (Blomberg-Martin et al., 2010) it is assumed that the source of information for DA neurons is various parts of the brain such as the lateral

habenula or parabrachial nucleus and not sensory stimuli directly (see Fig. 7).

In contrast, Galili et al. (2014) demonstrated a direct connection of sensory neurons (in this case, anterior cells of neurons in the central brain) after a heat shock with DA neurons and concluded that the neural pathways for different reinforcement signals are different but converge at DA neurons (including direct connection with sensory neurons). Hattori et al. (2017) studied whether activity in the PPL1 alpha '3 compartment is a consequence of primary stimulation of MBON; MBON stimulation did not increase DAN activity in this compartment, suggesting that the primary information did not come from MBON in this recurrent circuit.

So where does DAN obtain the information? Do they acquire information from sensory organs? Or from the first association centres (AL or KC)? Or is everything the result of feedback from MBON? Or is there another system in the game? Or they do not obtain the information directly, but at some point in the data processing, they are 'prompted' by other parts of the brain for an extraordinary reaction? It is quite possible that all eventualities are applied in different parts of the brain concurrently.

### 3.5. Dopamine and arousal

Another possible role of dopamine in the *Drosophila* associative learning process is not through reward function, but on arousal (Andretic et al., 2005; Ueno et al., 2013; Ueno and Kume, 2014), and on modulating the level of responsiveness (Kume et al., 2005; Crocker et al., 2010; Van Swinderen and Andretic, 2011). In this model, dopamine does not directly stimulate the reward system, but causes a decrease in the arousal threshold for a given environmental stimulus; associative learning is then applied in the desired direction (Krashes et al., 2009). This model still implicates the strengthening of associative learning, but without the need to use the reward function itself.

The role of dopamine in arousal functions or in alerting of new events has already been described in mammalian brain. DA can raise attention in any new situation, generally regulates the response to anything worthy of attention, but can also selectively raise attention based on the specific part of the network used. An unexpected sensory impulse evokes the activity of 60–90 % DA neurons in the ventral tegmental area and substantia nigra (Blomberg-Martin et al., 2010). DA neurons respond with phasic activity to an external alerting stimulus; 25 % of monitored DA neurons in monkeys responded to a new stimulus, but only 9% of cells responded to a repeat stimulus (Schultz et al., 1993). Both reward and arousal functions can coexist in dopaminergic cells. Fiorillo et al. (2013) describes a two-component response for DA cells reaction: the first when a new stimulus appears regardless of the reward, the second when a reward is present.

In *Drosophila*, genetic depletion of dopamine decreased arousal (Riemensperger et al., 2011). An increase in dopamine levels by dDAT mutations caused an increase in arousal (Kume et al., 2005; Ueno and Kume, 2014), as did methamphetamine, which primarily blocks DAT (Andretic et al., 2005). Hattori et al. (2017) described the role of DA in decreasing/increasing alert (alpha'3 DAN increases/decreases attention to olfactory stimulus according to novelty, thus a new odour significantly increases MBON activity in alpha'3 compartment and activity decreases rapidly with a repeat odour). Huang et al. (2018) reported that mechanical stress led PPLalpha3 DAN to augment arousal. DANs play an important role in the wakefulness and sleep management of *Drosophila* (Lebestky et al., 2009; Sitaraman et al., 2015a, b), and dopamine is attributed to the wake-promoting effect. Attention by alertness and attention targeted to the external stimulus are likely to be two distinct processes. Lebestky et al. (2009) distinguishes 'sleep-wake arousal' from 'environmentally induced arousal', which is of interest in our context (reward function). According to Lebestky et al., the control of environmentally induced arousal happens outside the mushroom body (in the ellipsoid body, in the central complex, in PDF-circadian pacemaker

cells). Moreover, like the above-mentioned dependency of the reward value on the types of MBON that process the information from DAN, which MBON class processes the information has important effects on arousal — Sitaraman et al. (2015b) reported that cholinergic MBON were sleep-inducing, while glutamatergic MBON mediated wakefulness.

The idea is that not only dopamine, but also the individual parts of the MB network are multifunctional and the arousal and reinforcement/reward functions run in parallel (assuming these functions can be distinguished in reality).

At present, it can be considered that the dopaminergic system is used in *Drosophila* associative learning in a manner similar to that in 'wanting' and 'learning' components in mammals, such as in designating an environmental stimulus as rewarding or in the motivation to obtain the reward. The DA system is a key system for creating the drive, for increasing the activity of the organism and targeting these activities to achieve the desired result. In experiments, it often manifests with increased activity, alertness, arousal, and locomotion. Logically, the second component, 'liking', should be associated with subdued activity and locomotion, arousal suppression, satiation and silence. Reward is thoroughly studied in context of the effects of drugs. The 'wanting' component corresponds more to the stimulant effect, while the 'liking' component to the opioid effect. If this idea is correct, the reward function of *Drosophila* in the sense of 'liking', would be evidenced not in its behaviour of searching for food but in no longer acting, due to satiety. It appears that experiments to date that confirm reward by increased activity do not generally describe 'liking', but instead describe 'wanting'.

#### 4. Neurochemical systems of the 'liking' component in *Drosophila* — endogenous opioids and endocannabinoids?

'Liking' in mammals is now attributed to neurochemical systems other than dopamine, particularly to the endogenous opioids and the endocannabinoid system. Hedonic hotspots in the mammalian brain (centres of the component 'liking'), whose stimulation leads to reinforcement and amplification of the hedonic component in natural reward sources, are in particular in the nucleus accumbens, ventral pallidum, nucleus parabrachialis, orbitofrontal cortex and insula (Berridge, 2009; Berridge and Krügelbach, 2013; Castro and Berridge, 2014; Berridge and Krügelbach, 2015; Castro and Berridge, 2017).

Pecina and Berridge (2005) reported that  $\mu$ -opioid activation (by DAMGO) at a location sized approximately 1 mm<sup>3</sup> in the rostrocaudal medial shell nucleus accumbens enhances the hedonic impact of sweet sugar. Interestingly, elsewhere in the medial shell NAc, the same receptors minimize the 'liking' effect and act like a 'coldspot'. Almost all other core and shell nucleus accumbens sites increase 'wanting' (Pecina and Berridge, 2005; Castro and Berridge, 2014).

These reports lead to the conclusion that the main tool of the hedonic component of reward is not dopamine (which apparently participates in the motivational component in 'wanting', without a direct effect on 'liking'), but endogenous opioids (Berridge and Krügelbach, 2015). However, there may be concerns about the way in which 'liking' reactions are determined in these studies: the investigated physiological manifestations (orofacial reactions in particular) are not necessarily manifestations of 'liking' component. It also remains a question why the amplification of the hedonic component does not lead to silence of the organism (in the way that opioid receptor stimulation results in pain relief or opioid drug administration leads to general psychosomatic depression), but rather increases food intake in experimental settings. Gosnell and Levine (2009) offer a possible explanation that opioids reduce the activity of another system of satiety, as in the oxytocin and melanocortin system, for example. However, the role of the endogenous opioid system in the reward function of mammals is generally widely accepted (e.g. Le Merrer et al., 2009; Gosnell and Levine, 2009, including an overview of studies demonstrating the role of opioids in hedonic type of food intake).

Later, endocannabinoids (anandamide and 2-arachidonoylglycerol) began to be considered key endogenous opioids that have a role in the

'liking' component. Evidence that endocannabinoids positively influence the reinforcing effects of natural rewards and drugs, and that they influence the mesolimbic DA system by direct modulation of CB1-receptor or indirectly through other signalling systems, has been summarized by Parsons and Hurd (2015) as well as Solinas et al. (2008). The works of Berridge's team describe the effect of endocannabinoids in a hedonic hotspot in the rostromedial subregion of the medial shell nucleus accumbens (anandamide increases the 'liking' reaction to the sweet taste of sucrose, Mahler et al., 2007; Mitchell et al., 2018).

Subsequently, another signalling system that affects on hotspots was described — orexin signalling (orexin activity in the nucleus accumbens hotspot Castro et al. (2016) in the ventral pallidum Ho and Berridge (2013), in the orbitofrontal cortex and insula Castro and Berridge, 2017). Orexin induces the 'liking' reaction, but is believed to be a tool by which the hypothalamus alters the hedonic impact depending on the state of homeostasis.

The relationship of the endocannabinoid and opioid systems with reward, as well as the mechanisms of action of these systems on mammalian dopamine circuits (independently of the concept of the aforementioned hotspots) have repeatedly been described (e.g. Wenzel and Cheer, 2018). Let us assume that stimulation of the opioid and endocannabinoid systems leads to (stronger) pleasure in mammals. Do *Drosophila* (and all other insects) have a similar neurochemical environment that could mediate the 'liking' as we know in mammals? Do endogenous opioid or endocannabinoid systems exist in insects? Neuropeptides are evolutionarily very old neurochemical systems, and are even found in non-bilateral animals (Diblastica) (Elphick et al., 2018). They occur abundantly in *Drosophila*, and the analysis of the *Drosophila* genome identified many neuropeptide systems associated with the G-protein receptor (mostly shared with mammals), but there is no opioid system among them (Caers et al., 2012; Jékely, 2013; Mirabeau and Joly, 2013; Semmens and Elphick, 2017; Elphick et al., 2018; Nässel and Zandawala, 2019).

When immunochemical studies were conducted in the 1980s and 1990s, there was increasing hope for evidence of an endogenous opioid system in invertebrates including insects (several older reviews Thorpe and Duve, 1990; Harrison et al., 1994; Nagabhusanam et al., 1995; immuno-chemical studies by Kream et al., 1980; Schoofs et al., 1988; Duve and Thorpe, 1988, 1989, 1990). Behavioural studies supported this view, revealing that the application of endogenous opioids elicited distinct physiological reactions in crustaceans (Reddy, 2000; Kumar et al., 2012), and insects (Zabala et al., 1984; Dyakonova, 2001), leading to the conclusion that insects must have opioid receptors. Swevers et al. (2005), by delivering the delta opioid receptor to the cell of *Bombyx mori*, verified that the cell is equipped with the necessary information cascade for the response triggered by opioid receptor activation. Santoro et al. (1990) used radio-labelled ligands to demonstrate  $\mu$ -like and kappa-like binding sites in *Drosophila*'s nerve tissue.

However, components of the opioid system were not found in the *Drosophila* genome (Mirabeau and Joly, 2013; Jékely, 2013). This is not so surprising, because the opioid system was not found in the honey bee genome either (Weinstock et al., 2006); accordingly, Dores et al. (2002) admits the possibility of secondary loss of endogenous opioids in Ecdysozoa. Larhammar et al. (2015) illustrate the development of opioids only within the evolution of vertebrates. Nevertheless, the absence of a component of the opioid system in the *Drosophila* genome does not exclude presence of an alternative neurochemical system. The above-mentioned works (e.g. Zabala et al., 1984; Dyakonova, 2001) demonstrating the active role of external opioids in insect behaviour control indicates the presence of some alternative mechanism.

Similar conclusions have been reached for endocannabinoid system in insects. Considering some evidence regarding the endocannabinoid system in non-insect invertebrates and research demonstrating the absence of the system in insects (Salzet et al., 2000; Elphick and Eger-tova, 2001; McPartland et al., 2005; Elphick, 2012), a secondary loss of endocannabinoid system in Ecdysozoa may be assumed.

Endocannabinoid ligands are metabolites of arachidonic acid and insects contain very little, if any, of this acid. The entire group Ecdysozoa may have undergone changes in phospholipid metabolism due to ecdysis (McPartland et al., 2001; Salzet and Stefano, 2002).

If neither the endocannabinoid nor the endogenous opioid systems are present in insects (which are believed to be mediators of the mammalian 'liking' component at present), we must conclude that the insect's reward system does not have the 'liking' component of reward as we know in mammals. Berridge et al. (2009) assumed that the proboscis extension reflex in *Drosophila* can be perceived as an objective manifestation of 'liking' (as a parallel to the orofacial pleasure reactions of the tested mammals), but this idea was disrupted by the finding of two interneurons in the ventral nerve cord, which regulate PER only through mechanosensory inputs by affecting satiety or taste sensations independently (Mann et al., 2013). Furthermore, the role of the TH-VUM dopamine neuron in the *Drosophila* sub-oesophageal ganglion that controls PER only by state of satiety, and not by taste or reward value, has been demonstrated (Marella et al., 2012).

Nevertheless, studies of the effects of drugs on *Drosophila*'s behaviour reveals interesting results. Kaun et al. (2011) describes the tendency for flies to be intoxicated with ethanol (they preferred an intoxicating dose rather than a low inefficient or high sedative dose), even at the cost of obtaining an electric shock. Similarly, Devineni and Heberlein (2009) describe that *Drosophila* prefers alcohol even with the presence of bitter quinine. These results highlight *Drosophila*'s preference towards experiencing a 'liking' effect.

Drugs, preferred by mammals, are also preferred by *Drosophila*. Several reviews describe studies that have investigated drug-related behaviour in *Drosophila*, e.g., Wolf and Heberlein, 2003; Heberlein et al., 2009; Kaun et al., 2012; Landayan and Wolf, 2015. By using *Drosophila* as a simple model organism of addiction development, researchers were able to examine genes that have altered expression during drug use or that may be implicated in the behavioural effects related to acute/chronic drug use (Atkinson, 2009; Rodan and Rothenfluh, 2010; Grotewiel and Bettinger, 2015; Park et al., 2017). Neurochemical systems mediating responses to drugs are interesting in the context of our topic, and dopamine plays an important role, not surprisingly (Bainton et al., 2000; Kong et al., 2010; Kaun et al., 2011; Freyberg et al., 2016).

An interesting conclusion is drawn by Shohat-Ophir et al. (2012). They found that sexually rejected (dissatisfied) males had a greater preference for alcohol than males after mating (satisfied) or males from control group. At the same time, they had the lowest dNPF level. The authors also verified a strong preference for odours associated with an increase in dNPF level, concluding that both alcohol and sex preferences were associated with an increase in dNPF and that dNPF/NPFR activation is rewarding per se (similar Shao et al. (2017) found that flies prefer the part of the experimental chamber that induces stimulation of NPF-expressing neurons, allowing them to conclude that NPF-expressing neurons induce pleasure). These results support the conclusion that other neurochemical systems, such as the dNPF system or the aforementioned orexin signalling system (Castro et al., 2016; Ho and Berridge, 2013; Castro and Berridge, 2017) could play a key role in the hedonic effect.

## 5. Other neuropeptide systems in the reward functions of *Drosophila*?

In the previous sections, we discussed the two key neurochemical systems for reward functions (important for its two core components 'wanting' and 'liking') in *Drosophila*. While the *Drosophila* brain has all necessities for 'wanting' (biogenic amines), structures for the 'liking' functions are not indicated. One might consider whether some insect neuropeptides perform the function that endogenous opioids or endocannabinoids perform in the reward system in higher animals. In this sense, publications on relativity of reward, specifically on the dependence

of reward on environmental context, are interesting.

Reward value is not absolute but varies according to environmental conditions, metabolic needs and physiological status. The main reason for changing the value of reward and its related associations/memories seems to be a change in nutritional and energy status. Several mechanisms by which metabolic needs regulate behaviour in *Drosophila* have been described (e.g. Pool and Schott, 2014; Su and Wang, 2014; Branch and Shen, 2017).

It is verisimilar that *Drosophila* always prefers the nutritional value of food over sweetness or other attractiveness (Burke and Waddell, 2011; Fujita and Tanimura, 2011; de Araujo, 2016). Even the attraction to nutritionally valuable foods is not absolute, but varies according to the internal state of the individual — the state of satiety/hunger (Colomb et al., 2009; Krashes et al., 2009). Attractiveness is driven by a change in the sensitivity of sensory inputs, another type of formed associative memory, other associative circuits used in the mushroom body, a change in memory retrieval, and possibly other mechanisms.

Ingestion of sweet sugar without nutritional value leads to the formation of the less stable short-term memory (STM) (Krashes and Waddell, 2008; Burke and Waddell, 2011), while ingestion of sugar with nutritional value improves not only STM but also protein synthesis-dependent long-term memory (LTM) (Yamagata et al., 2015). The effect of octopamine via OAMB receptors on dopaminergic neurons is important for the formation of STM on sweet sugar (the originally generalized role of octopamine in appetitive association learning and positive reinforcement in *Drosophila* has been reduced to the role in STM formation on sweet sugar only, through the action of dopamine neurons) (Burke et al., 2012; Huetteroth et al., 2015; Yamagata et al., 2015).

Various neuronal circuits and dopaminergic neurons projecting into different mushroom body lobes are used to form STM or LTM (Huetteroth et al., 2015; Yamagata et al., 2015; Das et al., 2016). Memory retrieval is state-dependent; the memory of a previous positive sugar reward is strongest in hungry flies, while it is inhibited in satiated flies. Krashes et al. (2009) described a neuronal mechanism that integrates motivation with a hunger/satiety state in *Drosophila*: dopaminergic neurons with dNPF receptors (MB-MP neurons, otherwise PPL1 cluster, thus aversive DAN) block memory performance in satiated flies — dNPF inhibits their activity (inhibition of inhibition results in release of appetite memory). Musso et al. (2015) found that one of these neurons have higher Ca oscillation following ingestion of nutritional sugar than sweet sugar and hypothesized that MB-MP1 has a role in post-ingestion signalling about the energy value of food. The state of satiety/hunger probably also influences which dopaminergic pathways control other behaviours. Landayan et al. (2018) described the involvement of PAM neurons in the motivation for feeding behaviour in hungry flies, and the involvement of PPL1, PPM2 and PPM3 neurons in satiated flies.

In *Drosophila*, dNPF/sNPF and insulin-like proteins are considered the main signals by which the metabolic state affects association processes, reward and memory. dNPF/sNPF have pleiotropic functions (review of the role of these peptides in invertebrates, including *Drosophila* Nüssel and Wegener, 2011; Fadda et al., 2019). The effect of sNPF on DAN has been mentioned above (Krashes et al., 2009). Root et al. (2011) described a mechanism by which hunger modulates food search behaviour at the 1<sup>st</sup> olfactory synapse in the antenna lobe glomerulus — the sNPF signal and the insulin-like signal are integrated at the olfactory sensory neuron, where they modulate the sensitivity of the olfactory input. Similarly, Inagaki et al. (2012) describe the influence of sugar level via the direct modulation of primary sensory gustatory neurons by dopamine and the DopEcR receptor.

sNPF also modulates the association process at the level of Kenyon cells. Knapek et al. (2013) identified sNPF in KC as the major co-transmitter (knockout of sNPF or their receptors led to impaired olfactory learning). The integration of the hunger/satiety signal at the KC > MBON synapse level is described by Tsao et al. (2018). The authors characterize 6 dopaminergic neurons, which receive multiple inputs of

hunger and satiety signals (HT, NPF, sNPF, ILP and allatostatin A). Groups of dopaminergic neurons PPL1- $\alpha$ 3, PAM- $\beta$ 2 $\beta$ '2a, PAM- $\beta$ '2a, PPL1- $\alpha$ '2 $\alpha$ 2 a PPL1- $\gamma$ 2 $\alpha$ '1 are positively regulated by hunger, while PPL1- $\gamma$ 1pedc is inhibited by hunger.

According to Wang et al. (2013), the appetitive odour-drive feeding is independent of the mushroom body; the key area responsible is the lateral horn and NPF receptors on dopaminergic DL2 cells — NPF mediates appetitive drive through dopaminergic signal. The work done on *Drosophila* larvae revealed that in the larval stage, the neural network is organized differently than in adults, and the role of NPF in association learning and reward seems to be more important (Rohwedder et al., 2015; Pu et al., 2018).

Allatostatin A is another candidate that could be involved in controlling insect reward function as mediator of 'liking'. Hergarden et al. (2012) described its 'satiety-like' effect: activation of allatostatin neurons inhibited feeding behaviour in adult *Drosophila* such as food intake and behavioural responsiveness to sugar. The authors also demonstrated that activation of allatostatin A neurons conveys satiety signals independent of detectable metabolic changes mimicking satiety. Chen et al. (2016) added that the activation of allatostatin-expressing neurons promotes sleep and decreases general activity (it can be assumed that satiety in the true sense of the word should be accompanied by a decrease in general activity).

## 6. Possible implications for the *Drosophila* reward system model

We have tried to summarize the current state of knowledge on the reward system, associative learning and related topics in *Drosophila*, with an emphasis on whether we can talk about insect reward systems using parameters similar to those present in mammalian reward systems — that is, whether it is possible to find at least a trace of the hedonic component in insects.

### 6.1. Is the complexity of the *Drosophila* brain sufficient for complex reward functions?

We can conclude that *Drosophila* has a very well-developed brain, even at the larval stage. It has an especially well-developed mushroom body, a structure that has key functions in associative learning. Some parts of the mushroom body exhibit qualities that appear to be sufficient to process information in dynamic, holistic and stochastic ways (at least as illustrated by the computational models of MB). It is likely that understanding the *Drosophila* reward system will allow development of a simple model widely applicable in the study of general reward problems, including those in mammals.

Unfortunately, the *Drosophila* brain network that processes olfactory perceptions from the outside world and associative learning, especially the mushroom body, is far too complex to accurately describe the flow and processing of information. The network is asymmetric with irregular architecture, and of varying developmental age; there are parts with straightforward processing of information, and parts working as a network, with combinatorial, probabilistic rules. Next to sites with strong activity of one neuron, there are sites with sparse activity of a random set of neurons. Some parts of the mushroom body work autonomously, and others work in strong connection with (almost all) other parts. An alternative to the common explanation of the information flow through a particular neurotransmitter and a particular synapse is the idea that co-transmission of several neurotransmitters in one neuron is more a norm than a peculiarity (moreover, a particular co-transmission may not be the primary state of a part of the network but may be a consequence of the network's experience with the environment, meaning that it may change dynamically). Moreover, one may also consider the multiplicity of different types of neuronal connections, where there exist not only the synapses with the most studied metabolic receptors, but also ionotropic synapses, gap junctions, en passant synapses, with the nonlocal effect of the neurotransmitter/modulator

and the possibility of nonlinear information flow. Another complication in understanding the work of the reward system is that the individual steps described in associative learning/memory/reward valuation (e.g., activity of individual neurons) do not take place as limited, isolated, processes, but rather as a longer operation (phasic and tonic, or combined), and as repeated impulses or recurrent cycles over time, oscillating in sparse sets, with summation of these activities (again, rather in longer periods of time and quite possibly with the summation of the sometimes chaotic form of the signal) and probabilistic outputs.

### 6.2. *Drosophila* has neurochemical 'activity-enhancing systems'

The neurochemical systems associated with the 'wanting' components, particularly the dopaminergic and in part, the octopaminergic systems, are now considered reliably documented in the *Drosophila* brain. This system works on similar principles as the mammal reward system. There are several interpretations of the roles of DA in the reward process ('DA as wanting', 'DA as a teaching signal', 'DA as an alerting signal'); however, a parallel occurrence of these functions is more likely. Thus, they work as 'activity-enhancing systems' — they enhance any activity to achieve the appetitive or desired.

Almost all *Drosophila* reward studies are focused on 'wanting' or other forms of reinforcement. Active 'wanting' is well documented (e.g., Lowenstein and Velazquez-Ulloa, 2018), but studies on the possible aspect of 'liking' in *Drosophila* are lacking. DA, or in some cases, OA, has a role in this search for the desirable. This model is fully consistent with the understanding of the role of DA in humans and mammals (there has been a shift from the attributed total reward mediation to the 'wanting' component). Active search for nutritionally valuable foods with increased activity and arousal can be — besides the 'wanting' function — controlled by mechanisms other than the reward function, for example, by innate mechanisms or by internal physiological imbalances such as sugar levels. Whether in the end the optimal level of sugar in the haemolymph brings a pleasant perception (and conversely, a lower sugar level an unpleasant perception) remains unknown. Thus, the 'wanting' component is not a necessary part of the reward process. Therefore, it is more accurate to talk about an activity-enhancing system rather than a 'wanting' system.

### 6.3. The presence of 'activity silencing systems' in *Drosophila* is still questionable

The systems of endogenous opioids and endocannabinoids associated with the hedonic component of reward in mammals are unlikely to be present in *Drosophila*. However, one cannot excluded the possibility that neuropeptide systems other than endogenous opioids also play a role. The original studies demonstrating the hedonic role of endogenous opioids in mammals (Pecina and Berridge, 2005; Berridge, 2009; Berridge and Kringelbach, 2015) mentioned this possibility. The authors assessed the hedonic effect of opioids by increasing behavioural activity of animals (to enhance feeding), but satiety should lead to silencing of activity. There is thus room for reflection on the key role of yet another neurochemical system, for example of allatostatin A (Hergarden et al., 2012; Chen et al., 2016; Yamagata et al., 2016).

Evidence of the absolute superiority of the homeostatic satiety over sugar sweetness may indicate, inter alia, that the 'liking' aspect in *Drosophila*'s reward is not included and that the *Drosophila*'s reward process is driven only by energy and nutritional needs. Alternatively, 'liking' could only be associated with the attractiveness of sweet sugar and formation of STM with full subordination to homeostatic needs. Another possible interpretation could be that DANs do not respond to reward, but to homeostatic needs (the opposite statement would be greatly supported by a study that shows *Drosophila* would prefer a pleasant taste over an unattractive, but nutritionally valuable diet). Thus, the ultimate goal of reinforcement is homeostatic satiety (expressed primarily by the fulfilment of some metabolic parameters). However, *Drosophila* also responds

strongly to other sources of reward, which will not impact metabolic parameters or homeostatic satiety (e.g., the effects of drugs, [Devineni and Heberlein, 2009](#); [Kaun et al., 2012](#)).

#### 6.4. Hierarchy of reinforcement levels

Hypothetically, we can build a hierarchy of reinforcement levels by the processing of a particular stimulus and by achieving an appropriate response to the stimulus:

- 1) Cellular level: even one cell is likely capable of making a minimal choice between desirable and undesirable and even of favourisation of the chosen solution in the next exposure. This can be visible in bacterial cells ([Koshland, 1980](#)), but also in single (sperm) cell of multicellular animals ([Eisenbach, 2004](#)) or neuronal ([Debanne et al., 2019](#)) cells.
- 2) Reinforcement as a quality of the network — reinforcing the simultaneous processing of two stimuli, or reinforcing a specific path of information to a specific target behaviour, can only happen on the basis of general network characteristics (reverberation, oscillation, sparse activity patterns, Hebbian learning). The network itself without the participation of complementary systems mentioned below can lead to results that imply a form of associative reward learning.
- 3) The use of a ‘complementary’ system that adds importance to the network’s response to a given stimulus — diminishes interest in other stimuli, marks the current stimulus as significant, boosts the neuronal pathway processing the given stimulus, or adds to this neuronal pathway an activity of another neuronal pathway, and extends the processing of a particular stimulus to other brain areas (monoamines and especially DA are candidates for this role).
- 4) The use of a ‘satisfaction system’ that complements previous mechanisms by achievement of general satisfaction and silencing, including attenuating the activity of alert, stress, algic, motor or sensory systems, thereby eliciting a state perceived probably as very strongly hedonic. Several specific neuropeptide systems could be used in this function. Their role could be derived from the homeostatic, metabolic satiety by generalization of this role through the suppression of the aforementioned stress, alert, algic, motor and sensory systems (each of these systems may have its own silencing

neuropeptide) to the general ‘meta-satiety’ (opioid and endocannabinoid). These systems could be termed ‘activity-silencing’.

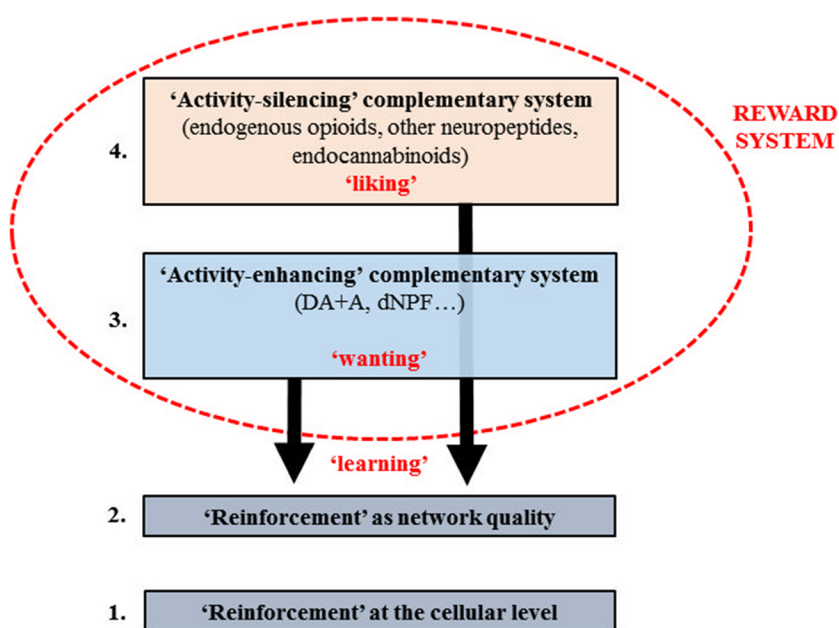
It is perhaps a common feature of evolutionary advanced reward systems, with their asymmetric and hierarchical architecture, that they contain all of these levels concurrently and simultaneously ([Fig. 8](#)).

In *Drosophila* for the time being, there seems to be no evidence of the presence of this level — for the presence of generalized ‘activity-silencing’ and ‘meta-satiety’ inducing level. Or, this level would be better divided into the level of silence of a particular active brain system (stress, alert, algic, etc.) and the level of generalized silencing, for the moment distinguished from metabolic satiety by calling ‘meta-satiety.’ We also assume that if *Drosophila* has signs of activity-enhancing systems, it could also have a tool for stopping this activity, and some works on neuropeptides demonstrates the presence of these systems. However, the activity can also be stopped by its time constraint, and the achieving of optimal parameters can only be evaluated sensually over and over again in the form of: if any physiological parameter is low, a time-limited drive starts an action to minimize parameter disbalance, and this is repeated until the arousal and energy are sufficient or until the imbalance is balanced. In any case, the organization of ‘satisfaction-systems’ in *Drosophila* (and in whole insects) can be based on completely different principles than in higher animals (although other neurochemical systems are often evolutionarily conservative).

#### 7. Conclusions and future directions in the study of the reward system in *Drosophila*

We are aware that our review brings more questions than answers. It would be interesting to design and conduct experiments that would help investigate the ‘liking’ component in the reward process. In this sense, the preference of stimulus without any physiological value for *Drosophila* (e.g., drugs) may not be sufficient to test solely by increased activity. It might be interesting to test the mechanisms that underlie not in increasing activity but in silencing it. We speculate that achievement of the reward will lead to satiation and cessation of the activity. Further, another interesting subject of investigation is whether neuronal systems, whose original function is to achieve satiety after fulfilment of a physiological need, are re-used for hedonic reward functions.

The existence of a reward system in insects has significant practical implications; the insect model’s simplicity facilitates the study of the



**Fig. 8.** A hierarchy of basic levels at which the processing of a particular stimulus and the achievement of appropriate response to this stimulus can be reinforced. The arrows indicate the direction of ‘learning,’ pointing from reward levels back to the network level. ‘Learning’ probably does not take place directly in complementary systems, but rather complementary systems influence the learning of networks. Hypothetically, the mechanisms of the reinforcement of stimulus processing and of achieving an appropriate response to this stimulus may occur in parallel in a given part of the network. The network may apply to stimuli that requires processing at different levels. The network can use only one level or all levels concurrently.

behaviour of the neural network and the translation of the results into human brain research. Finally, the fact that insects presumably have a relatively advanced reward system may have philosophical implications. Pleasure and displeasure can be seen as nuclear primary emotions, and insect learning, including relatively complex forms of learning in some cases, is undoubtedly considered a nuclear form of cognitive function. In other words, it is possible to attribute to insects at least the basic forms of higher brain functions.

Given that *Drosophila* serves as a simple model for studying the principles of the human brain, let us conclude with a little comparison; the reward system of insects does not seem to have a reward silencing effects (opioid and endocannabinoid phase) but the human reward system has a peculiar awareness of this ‘opioid phase’ as well as a focus on delayed gratification, leading humans to strive towards accessing this pleasure consistently over time rather than pursuing immediate, transient pleasures. This may mean that both paths lead to a massive search for reward resources. So one question remains: are these phenomena related to the evolutionary success of both groups of animals?

A number of behavioural studies conducted in *Drosophila* clearly confirm that the fly mushroom body network is capable of associative learning, although the detailed mechanism remains unclear. One of the prerequisites for associative learning is the ability to discriminate the appetitive from the aversive — which, however, is also requirement of the reward function. On the other hand, this ability does not necessarily imply the presence of the reward itself as we assume it in mammals, including the hedonic component. Further, it is possible to imagine associations caused by different mechanisms: a simple ‘learning’ mechanism of a single cell, an association mechanism inseparable from network properties, or a mechanism of support of associative processes by a secondary system. This system may utilise DA or another neurotransmitter and may allow/initiate/strengthen the associations without being a direct bearer of the reward. Another mechanism may utilise a system conveying information about generalized satiety, satisfaction, and reward in the sense of ‘liking’; in this form, reward could already be a form of abstract operation. We suppose that further investigation of the insect reward function might provide more evidence of the ‘liking’ function, probably based on different neurochemical systems than in mammals.

## Acknowledgements

This study was supported by the project RVO 60077344 of the Institute of Entomology. The English grammar and stylistics were checked by the Editage Author Services.

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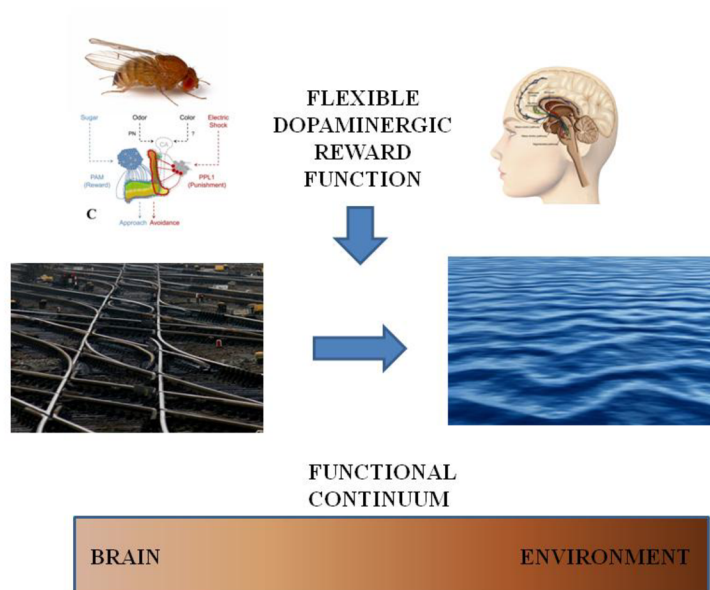
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# 4. KAPITOLA

## Článek 2 - Dopaminergní neurony mushroom body Drosophily: flexibilita neuronové identity v modelovém organismu?



Dvořáček, J., Bednářová, A., Krishnan, Natraj, Kodrík, D. (2022). Dopaminergic mushroom body neurons in *Drosophila*: flexibility of neuron identity in a model organism? *Neuroscience and Biobehavioral Reviews* 135, 104570. <https://doi.org/10.1016/j.neubiorev.2022.104570>



Contents lists available at ScienceDirect

## Neuroscience and Biobehavioral Reviews

journal homepage: [www.elsevier.com/locate/neubiorev](http://www.elsevier.com/locate/neubiorev)

Review article

Dopaminergic mushroom body neurons in *Drosophila*: Flexibility of neuron identity in a model organism?Jiří Dvořáček<sup>a,b,\*</sup>, Andrea Bednářová<sup>a</sup>, Natraj Krishnan<sup>c</sup>, Dalibor Kodrík<sup>a,b</sup><sup>a</sup> Institute of Entomology, Biology Centre, Czech Academy of Sciences, Branišovská 31, České Budějovice 370 05, Czech Republic<sup>b</sup> Faculty of Science, University of South Bohemia, Branišovská 31, České Budějovice 370 05, Czech Republic<sup>c</sup> Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, MS 39762, USA

## ARTICLE INFO

## Keywords:

Dopaminergic function  
Brain reward system  
Co-transmission  
Neurotransmitter switching  
Insect brain  
Neural plasticity  
Universality of neural function

## ABSTRACT

In classical neuroscience, Dale's principle postulates that neuronal identity is conferred by the specific neurotransmitter that it releases. However, the brain might be more tractable to specific situations regardless of specific specialisation which may contradict this principle. Hence, this constrained approach of how we perceive and study the nervous system must be revisited and revised, specifically by studying the dopaminergic system. We presume a relatively flexible change in the dopaminergic system due to neuronal activity or environmental changes. While the parallel between the reward system of mammals and insects is generally well accepted, herein, we extend the idea that the insect nervous system might also possess incredible plasticity, similar to the mammalian system. In this review, we critically evaluate the available information about the reward system in vertebrates and invertebrates, emphasising the dopaminergic neuronal plasticity, a challenge to the classical Dale's principle. Thus, neurotransmitter switching significantly disrupts the static idea of neural network organisation and suggests greater possibilities for a dynamic response to the current life context of organisms.

## 1. Introduction

The classical view of the nervous system function is relatively descriptive and static. To date, significant progress has been made in describing specific connectomes. In many parts of the neural network, the connections between neurons are described relatively accurately anatomically. Individual neurons in these connectomes have been identified, and their neurotransmitter identity and composition, transmitter signalling pathways, and intracellular information cascades in neurons have been described. Although we know that the extracellular and intracellular pathways of information are complicated and confusingly branched and interconnected, we tend to assume that it is only a matter of time and depth of study, when this complexity will be deciphered (in the form of complex but still describable processes, anatomical connections, and information processing pathways). Furthermore, we assume that most (if not all) parts of these brain structures are specialised for a specific function; the basic elements of most brain structures (if not all) are fixed from the developmental period; and their development and final connection are complicated and specifically controlled. The plasticity of brain structures only completes or unfolds what is otherwise constituted as hardwired.

The neurotransmitter type has a special position in this manner of understanding the function of brain structures. It is an exaggeration to say that anatomical structures, including the entire connectome, are viewed as pathways of a particular neurotransmitter to its specific target (receptor). Thus, the description of the neurotransmitter involved and its receptor appears, under certain circumstances, as an obvious answer to many questions, from the description of the function of specific connections in the network to the explanation of the aetiology of mental disorders.

Neuronal identity is predominantly described by the present neurotransmitter. One of the basic postulates of classical neuroscience, Dale's principle states that the neuronal identity is given by a specific neurotransmitter: each neuron releases a single classical transmitter (although the original formulation of this principle was not by Dale, but by Sir Eccles, and had a different meaning: In conformity with Dale's principle that the same chemical transmitter is released from all the synaptic terminals of a neurone (Eccles et al., 1954)). In the model, where the identity of the neuron is given by a classical neurotransmitter, although plastic changes occur that modify the function in response to the environment or experience, these changes do not change the (neurotransmitter) identity of the neuron.

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<https://doi.org/10.1016/j.neubiorev.2022.104570>

Received 8 December 2021; Received in revised form 3 February 2022; Accepted 3 February 2022

Available online 4 February 2022

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Studies in the past decade have shed more light on this paradigm. On the one hand, ideas are formulated about 1) greater universality of brain functions (i.e. less specialisation in a particular function (Anderson, 2010; Brezina, 2010; Marder, 2011)); on the other hand, it is amply documented that 2) the co-transmission of more transmitters in one neuron is more common than exceptional, and neurons are capable of neurotransmitter switching, changes in transmitter identity, and that the environment can trigger such significant changes. Additionally, the quality of the entire system cannot be fully understood from the description of the network elements (Bargmann and Marder, 2012).

The current view of the brain thus shifts to the idea of a network rather universal than fully specialised, with qualities arising in the complexity of interconnection imbued not only with the potential for plastic changes but with lasting flexibility and dynamism as the main feature of the function, with fluidity allowing flexible and rapid response to changes in the complicated external and internal environments of the organism (Brezina, 2010).

The fruit fly (*Drosophila melanogaster*) is a simple model for studying numerous processes in the mammalian (and human) physiology and pathology (see e.g. our review Bednářová et al., 2017), but in this review we chose the opposite procedure: for studying *Drosophila*, we took inspiration from the mammalian brain physiology. In the *Drosophila* reward system, the quality and possibilities of reward processing are derived from the quality of the reward network (and, among other things, from the number and quality of dopaminergic cell interconnections), and hardwired structures limit the reward quality. This raises the question that whether the quality of the hardwired reward connectome is not only limited, but the reward does not change the connectome at the same time and it causes a change of this type: the more reward the organism gains, the more the connectome is adapted to acquire it. The principles of neural network organisation are developmentally conserved among organisms; dopaminergic neurons (DANs) in reward functions have similar properties in *Drosophila* and mammals (Perry and Barron, 2013; Waddell, 2013; Dvořáček and Kodrčík, 2021). Therefore, we briefly looked at how the neurochemical identity (and the possibility of flexibly influencing this identity by the environment) of dopaminergic neurons is understood today in the mammalian reward system. A reward system that is plastically responsive to the environment would be more appropriate for complex and changing environmental conditions than a reward system with a constant neurochemical identity. Our idea is simple: when many neuronal systems, including motor ones, show the ability of transmitter plasticity, why should the key system for adaptation to the outside world (reward system) be omitted?

## 2. Dopaminergic reward function in *Drosophila* and transmitter plasticity

### 2.1. *Drosophila* reward system - Basic neurochemical identity

In general, the brain reward system, as a tool for labelling biologically important stimuli with attractive or aversive signs and increasing the likelihood of behaviour leading to preference/aversion, is a complex of functions rather than a unified brain system. The study of insect reward systems has been inspired by those in mammals and shares some concepts with them. It seems practical to divide the complex of reward functions into components 'liking', 'wanting' and 'learning', where 'liking' is the hedonic component of the reward itself (pleasure), 'wanting' is the motivation to obtain the reward (drive), and 'learning' are classic and instrumental associations and cognitive representations of the reward (Berridge, 2008; 2009). Although some studies have suggested the possible presence of the 'liking' component in *Drosophila* (Kaun et al., 2011; Devineni and Heberlein, 2009; Shohat-Ophir et al., 2012), we considered 'wanting' and 'learning' components only, which are documented in *Drosophila* and in which dopamine is involved as a key neurotransmitter, similar to the reward system in mammals (Waddell,

2010; 2013; Oswald and Waddell, 2015; Scaplen and Kaun, 2016; Kaun and Rothenfluh, 2017; Cognigni et al., 2018; Bednářová et al., 2017).

The *Drosophila* reward system is described primarily in connection with olfactory associative learning and is because *Drosophila* effectively discriminates between rewarding and aversive environmental stimuli and clearly adjusts behaviour to seek reward and avoid punishment. Stimulus or behaviour marked with reward value is the subject of associative learning and the formation of short-term and long-term memory (Dvořáček and Kodrčík, 2021).

The key brain structures of *Drosophila* olfactory associative learning are found in the mushroom body (MB). They include connections of antennal lobe projection neurons with dendrites of Kenyon cells (KC) in the calyx of the MB, connections of KC axons with mushroom body output neurons (MBON) in the MB lobes, and connections of the DAN axon with the presynaptic part of the KC vs. MBON synapse. Information about the positive/negative value in associative learning represents an unconditioned stimulus, which, according to the classical idea, is represented by DAN activity - the association of this reward with odour as a neutral stimulus occurs at KC vs. MBON synapses (Waddell, 2010; Schwaerzel et al., 2003; Riemensperger et al., 2005).

This straightforward idea is complicated by the description of feedback loops and recurrent circuits in MB and synapses other than those just mentioned (Aso et al., 2014a; 2014b; Ichinose et al., 2015; Takemura et al., 2017; Felsenberg et al., 2017); however, the activity of DAN is still considered crucial for being designated a reward response circuit.

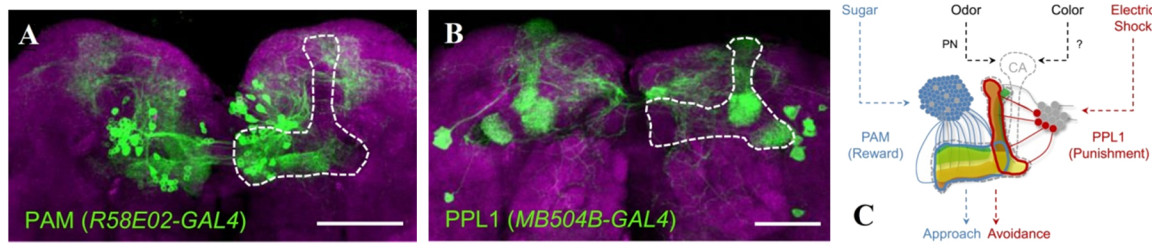
Currently, the identity of this part of the brain associated with reward functions in adult *Drosophila* is clearly and unambiguously determined by the architecture, neurotransmitters involved, and very precisely described connections at the cellular level. However, there is a tendency (and without much exaggeration) to compute and map the involvement of each neuron (Aso et al., 2014a; 2014b; Takemura et al., 2017; Cohn, Morante and Ruta, 2015; Davie et al., 2018). DANs that project into MB lobes (protocerebral anterior medial cluster and protocerebral posterior lateral 1 cluster, see Fig. 1) represent a relatively precise group of 130 cells, which is heterogeneous in its output to different MBONs (Aso et al., 2014b) (describing 20 types of DAN), in reinforcement valences (positive/negative/bidirectional), and in types of memory in which they participate (Oswald and Waddell, 2015; Mao and Davis, 2009; Aso et al., 2012; Burke et al., 2012; Huetteroth et al., 2015; Yamagata et al., 2015; Perisse et al., 2016; Handler et al., 2019), but the neurochemical identity of DAN is always fixed.

In *Drosophila* DAN, it is assumed that they are involved in reward functions that rely exclusively on dopamine alone. According to one of the main ideas, dopamine in olfactory association learning is intended to modulate the KC vs. MBON synapse and change their synaptic weight (Schwaerzel et al., 2003; Riemensperger et al., 2005; Claridge-Chang et al., 2009). Here, dopamine can represent the reward prediction error (Schwaerzel et al. 2003), increase the level of arousal (Krashes et al., 2009), the level of responsibility (Kume et al., 2005), or directly carry information about the reward value (Waddell, 2013).

### 2.2. Slow dopaminergic signalling

Dopamine signalling is probably not very suitable for directly transmitting information about the reward value/potential in the current situation (especially for its action via G-protein coupled receptors [GPCR] and for the possibility of volume transmission). It proceeds relatively slowly due to a complicated signalling pathway with many biochemical intermediate steps (cAMP as a second messenger, protein kinase A, phosphorylation of threonine-34 and dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32,000 [DARPP-32] and possibly other substrate proteins, DARPP-32-PPi cascade), has a prolonged effect (Greengard, 2001) and lacks spatial and temporal specificity (in mammalian DAN, Lapish et al., 2006; Lapish et al., 2007).

The temporal and spatial specificity of the dopamine signal is disabled not only by a dominantly prolonged effect but also by the



**Fig. 1.** Dopaminergic neurons in the *Drosophila* brain (Vogt et al., 2014). A, B - Visualization of two main sets of dopaminergic cells projecting to the mushroom body (PAM and PPL1 clusters), dashed line - the position of the mushroom body, scale bars - 50  $\mu$ m, C - scheme of basic bidirectional modulation of reward / punishment value by different groups of dopaminergic neurons in the mushroom body and formation of appetitive /aversive memories in associative learning. CA - calyx of mushroom body, PN - projection neurons, PAM - protocerebral anterior medial cluster, PPL1 - protocerebral posterior lateral 1 cluster.

complexity of action through various receptors at a relatively small site [opposite action of dopamine D1-like receptors and D2-like receptors on membrane excitability (Gerfen and Surmeier, 2011)], due to the possibility of volume transmission and diffusion from the synaptic signal to the neighbouring synapse (Smiley et al., 1994). The *Drosophila* genome encodes four types of dopamine receptors (Dop1R1, Dop1R2, DopR2, and DopEcR), all of which belong to the GPCR family. All four types are found in the KC of the MB (Croset, Treiber and Waddell, 2018; Kondo et al., 2020); 24% of KCs express all these receptors simultaneously (Gerfen and Surmeier, 2011). Different dopamine receptors are located in different parts of the membrane of the same neuron. Thus, one neuron can simultaneously perform different dopamine functions (Kondo et al., 2020).

For completeness, we add that the ability of dopamine to conduct a fast signal is not completely ruled out - in some animals (e.g. *Aplysia*, Mollusca), dopamine can exert a rapid excitatory postsynaptic potential and apparently utilise ligand-gated synaptic signalling (Díaz-Ríos and Miller, 2005). Another complication of dopamine function may be the relative independence of the dopamine signal on total dopaminergic cell firing (Berke, 2018). In his work on the role of dopamine in mammalian learning and motivation, Berke stated that dopamine release can be controlled locally, independent of the cell body firing.

In the previous section, we briefly described the basic scheme for olfactory learning in *Drosophila* (the information about conditioned stimulus (CS) = odour goes through projection neuron in antennal lobe to KC in MB and then to MBON, information about unconditioned stimulus (US) = reward goes through DAN to MBON, where it should occur to the convergence of both signals). The entire information part of the CS is led by fast-acting neurotransmitters, and the US path, at least in the final part, is led by a slow-acting neurotransmitter (dopamine). Thus, it is obvious that if the US information path is not faster for some other reason, the signals cannot meet at the same time at KC-MBON synapses, which can lead to Hebbian learning, which should dominate in associative learning according to the current body of knowledge (in computational models Nowotny et al., 2005; Delahunt et al., 2018).

This slow nature of the dopamine signal and low temporal/spatial sensitivity may be sufficient to attribute reward value to situations of longer duration, but for rapid and early reward signalling or its prediction, an isolated neurochemical identity of DAN is probably not sufficient.

### 2.3. Co-transmission in general

Currently, there is a body of knowledge about the function of DAN in the mammalian reward system, which can serve as a source of inspiration for new experimental approaches to broaden our knowledge of the *Drosophila* reward system. As a basic network mediating reward function in mammals, we still consider the mesolimbic dopaminergic system with a key position of DAN in the ventral tegmental area (VTA), although there has been a shift in understanding of the role of these neurons. Dopamine is no longer the major hedonic neurotransmitter

[this role is attributed today to endogenous opioids and endocannabinoids (Berridge, 2009; Berridge and Kringelbach, 2015; Mahler et al., 2007; Mitchell et al. 2018)], but it participates in the motivational drive, the 'wanting' component of reward (Berridge and Kringelbach, 2015) mediating reward prediction error information (Schultz, 1997), or increasing alertness (Blomberg-Martin et al., 2010).

Presently, in the mammalian reward system, DAN does not use dopamine alone. The main role in the reward mentioned above still belongs to the DAN but probably no longer (only) to dopamine.

Co-transmission in vertebrate neurons has been known for several decades. For example, in 1976, Burnstock et al., (1976) formulated the possibility that co-transmission in some neurons in some species is a common phenomenon and that 'each neurone, being a cell supplied with a complete set of genes, possesses the potential ability for synthesising the complete enzymatic machinery for all transmitter substances.' Co-transmission is subsequently described as a phenomenon common in mammalian neurons, not only in the combination of classical neurotransmitters and neuropeptides (Nusbaum et al., 2001; Nusbaum et al., 2017), but also in the case of multiple transmitter signals of several classical neurotransmitters (Black et al., 1987; Hnasko and Edwards, 2012; Svensson et al., 2019). These reviews also describe possible forms of the relationship between two or more co-transmitters in one neuron and possible mechanisms of storage, transport, and modulation. Variants of these relationships are described, among other things, by the distinction between the terms 'co-localization', 'co-release', and 'co-transmission' (Svensson et al., 2019; Vaaga et al., 2014). In this review, we have no ambition to focus on intra-neuronal and molecular processes; for simplicity, we used the term co-transmission as a term summarising all these processes, regardless of whether it is a synaptic transmission of information or whether they are located in common vesicles of the axon.

Svensson et al. (2019) considered co-transmission, even as a norm. In terms of co-transmission, neurons can be divided into three types: (a) neurons combining two fast-acting transmitters, (b) neurons combining fast-acting transmitters with neuromodulators (peptides, ions, or other small molecules), and (c) neurons combining fast-acting transmitters and slow-acting monoamines (Vaaga et al., 2014). The representation of transmitters in a neuron is not constant or static. Black et al., (1987) spoke of transmitter plasticity ('critical molecular processes appear to be in constant flux, influenced by environmental stimuli and conditions') not yet in the form of a neurotransmitter switching (see below) but in the response of transmitter levels to external stimuli.

Many recent studies have focused on co-transmission in mammalian reward systems, especially in the DAN of the VTA. The co-transmission of dopamine and glutamate in these neurons in developing and adult mammals (studies were performed in rats, mice, primates, including humans, in vitro and in vivo; dopaminergic characteristics are usually detected by the presence of tyrosine hydroxylase (TH), glutamate according to VGLUT2 transporter) is sufficiently documented (Lapish et al., 2006; 2007; Vaaga et al., 2014; Sulzer et al., 1998; Stuber et al., 2010; Dal Bo et al. 2008; Chuhma et al., 2004; Root et al., 2014; Sulzer and

Rayport, 2000; Barker et al., 2016; Mingote et al., 2019).

However, the relationship between these two transmitters remains ambiguous. Glutamate causes rapid excitation in asymmetric synapses and appears to act more slowly in symmetric synapses but is also released from axonal varicosities and is active extrasynaptically (Sulzer and Rayport, 2000; Zhang et al., 2015; Descarries et al., 2008). Both transmitters can be placed differently in the axon; some variants of location correspond more to an independent function, others to a closer co-transmission in the true sense (important review of phenotypic diversity in VTA Barker et al., 2016; Morales and Margolis, 2017). Briefly, both neurotransmitters can be in the same vesicles (Berrios et al., 2016), in the same axon in separate independent domains (Zhang et al., 2015; Kawano et al., 2006), or in the same axon terminals (Root et al., 2014).

Glutamate co-transmission is described only in a part of the DAN in the VTA, in which neurons express both glutamate and dopamine, neurons only with dopamine, and neurons only with glutamate (Root et al., 2016). Dual neurons (with co-transmission of glutamate and dopamine) are identified mainly by their output; they point to the medial shell of the nucleus accumbens (NAc) and to the prefrontal cortex (PFC) (Mingote et al., 2019; Poulin et al., 2018; Yamaguchi et al., 2011; Kabanova et al., 2015), or vice versa: input to PFC and medial shell NAc comes from VTA only from 'VGlut2- TH neurons' and 'VGlut2-only neurons' (Zhang et al., 2015; Yamaguchi et al., 2011). The mode of network involvement of VTA neurons according to neurotransmitter identity is shown in Fig. 2.

Notably, this neurotransmitter identity is not constant - at least it changes with age (Dal Bo et al. 2008; Bérubé-Carrière et al., 2009;

Mendez et al., 2008) or with context (Barker et al., 2016). Morales and Margolis (2017), in their review, recall evidence of TH + neurons in the VTA with differently expressed individual components of the dopamine signal, including evidence of TH + neurons without dopamine (Yamaguchi et al., 2015). Thus, we can speculate whether similar findings can be evidence of the neurotransmitter switching in VTA mentioned below.

Due to the different properties of their signalling pathways, both transmitters probably represent different functions in DAN: dopamine = prolonged slow action (Lapish et al., 2006; 2007), glutamate = fast short action (Chuhma et al., 2004). The relationship between dopamine and glutamate signalling pathways is described in detail by Greengard (2001). The signal during reward processing is probably biphasic. Lapish et al. (2006; 2007) assume that the reward prediction error signal carries glutamate; the onset of dopamine is too slow to transmit such information and has a considerable delay. The DAN of VTA respond to reward and its prediction with fast and transient glutamate signal (mediates temporally precise signal), dopamine tonically and in a prolonged manner modulates PFC networks, keeps the activity of this network alert in a given reward situation and informs the PFC network by increasing/decreasing the dopamine level about the accuracy of the prediction. Greengard (2001) summarised his exact description of the relationship between dopamine and glutamate signalling as follows: 'However, when one thinks of fast synaptic transmission as being the hardware of the brain, and slow synaptic transmission as being the software that controls fast transmission, the molecular basis by which nerve cells communicate with each other makes more sense'.

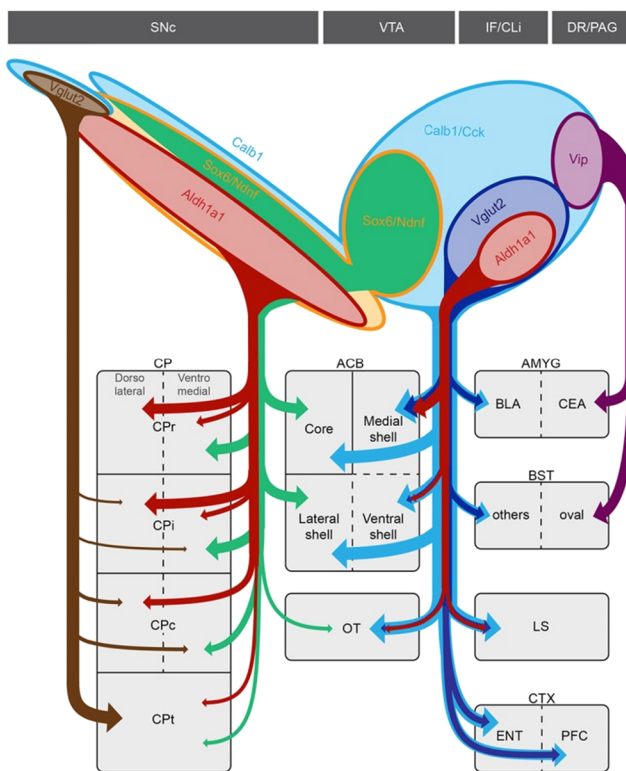
The situation will probably not be so simple; dopamine in the VTA neurons acts by many mechanisms; its role can be as a messenger and/or modulator, synaptic and/or extrasynaptic, independent of receptors [basic review of the role of dopamine in VTA neurons (Lapish et al., 2006; 2007; Mingote et al., 2019; Morales and Margolis, 2017; Trudeau et al., 2014)]. Particularly, Mingote et al. (2019) described the role of other transmitters in VTA neurons, especially gamma amino-butyric acid (GABA) (see Fig. 3) and, similar to Morales and Margolis (2017), emphasise that all major transmitters are involved in reward functions.

Despite the complexity associated with multiple functions and transmitters in the VTA, co-transmission of glutamate and dopamine in this reward region appears to be specific. Several studies have confirmed the absence of this co-transmission in other nearby regions containing DAN, such as the substantia nigra (Stuber et al., 2010) or termination in parts of the NAc other than the medial shell (Mingote et al., 2019).

#### 2.4. Dopamine neurotransmitter switching in general

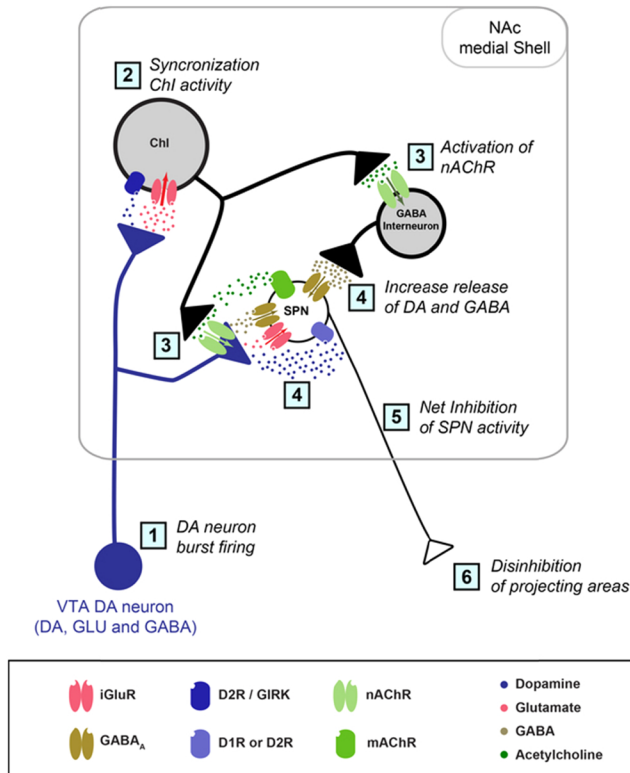
Under certain conditions, neurons are likely capable of changing neurotransmitter and neurotransmitter respecifications or switching. This phenomenon has been described in vertebrates several years ago (Spitzer, 2015; Li et al., 2020). Neurotransmitter switching significantly disrupts the static idea of neural network organisation and suggests greater possibilities for a dynamic response to the current life context of organisms. This is probably a common feature of neurons, although some neurons can be switched more easily than others (Li et al., 2020). They also point out that a switch can occur only in some parts of a neuron (e.g. at a nerve terminals), while another part of the same neuron (e.g. a cell body) has the original transmitter phenotype.

Neurotransmitter switching can be defined as '...an extreme form of neurotransmitter plasticity, wherein the expression of neurotransmitter genes or proteins is up-regulated from zero, that is, "switched on", or down-regulated to zero, that is, "switched off" (Aumann, 2016). Recently, Li et al. (2020) defined this with a simpler formulation: 'a form of brain plasticity in which an environmental stimulus causes neurons to replace one neurotransmitter with another, often resulting in changes in behaviour'. There is much evidence of neurotransmitter switching in mammalian neurons during neuronal development (Spitzer, 2015; 2017), but in this review, we mainly focused on evidence in the adult



**Fig. 2.** The mode of network involvement of VTA neurons according to neurotransmitter identity (Poulin et al., 2018) DAN with different neurotransmitter equipment have different connections in the mouse neural network. Abbreviations: ACB - nucleus accumbens, BLA - basolateral amygdala, BST - the oval nucleus of the bed nucleus of the stria terminalis, CEA - central amygdala, CP - caudate putamen (CPr = rostral, CPi = intermediate, CPc = caudal, CPt = tail), DR - dorsal raphe, ENT - entorhinal cortex, LS - lateral septum, OT - olfactory tubercle, PAG - periaqueductal gray, PFC - prefrontal cortex, SNc - substantia nigra pars compacta, VTA - ventral tegmental area. Subpopulations of DAN are described by different Cre drivers.





**Fig. 3.** The figure shows the hypothetical role of cotransmission in the nucleus accumbens medial shell in mice (Mingote et al., 2019). Abbreviations: Ach - acetylcholine, DA - dopamine, D1R - dopamine D1 receptor, D2R - dopamine D2 receptor, D2R / GIRK - dopamine D2 receptor coupled with G protein-activated inward rectifier potassium channels, ChI- cholinergic interneuron, ChR2 - channelrhodopsin, GABA - gamma aminobutyric acid, Glu - glutamate, iGluR - ionotropic glutamate receptor, mAChR - muscarinic receptor NAc - nucleus accumbens, nAChR - nicotinic receptor, NpHR - halorhodopsin, SPN - spiny projection neuron.

nervous system, especially on changes in the dopamine phenotype of neurons.

The possible flexibility in responses of DAN to external environmental cues is enabled by the activities that regulate the number of DANs, for example, by changing the neurotransmitter composition (Aumann, 2016; Spitzer, 2017). Tandé et al. (2006) reported that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism led to an increase in the number of TH-immunoreactive neurons in the striatum in 20–25-year-old macaques after 3 weeks. Furthermore, the authors suggested that it was probably a switch (they themselves used the term ‘phenotypic transdifferentiation’) from pre-existing neurons with the original GABA identity. Similarly, Velázquez-Ulloa et al., (2011) documented the formation of a dopamine phenotype from neurons with the co-transmission of neuropeptide Y and GABA (in amphibian larvae of *Xenopus laevis*). In neurons without GABA, this neurotransmitter change was not observed. In mice, an activity-dependent increase in the number of TH+ neurons was described by Aumann et al., (2011); (2013), who stated that the number of DANs is not fixed in the substantia nigra pars compacta (SNpc) but regularly increases/decreases in response to environmental stimuli. Tomas et al. (2015) described two elements of the environment leading to an increase/decrease of the dopamine phenotype in midbrain neurons of adult mice: continuous pairing of both sexes and environmental enrichment (EE) (in their conception an environment providing ‘environmental novelty and complexity relative to standard housing’). Similarly, Dela Cruz et al. (2015) noted an increase in TH+ in VTA rats after deep brain stimulation of the anterior nucleus of the thalamus.

Several studies have documented the switch in the dopamine phenotype due to a change in the length of the photoperiod. The switch in the interneurons of the hypothalamus of adult rats between the dopamine and somatostatin phenotypes in response to photoperiod length (short day photoperiod led to the formation of new dopamine cells) was described by Dulcis et al. (2013). The acquisition of a secondary dopamine phenotype in the neuropeptide Y neurons in the ventral suprachiasmatic nucleus in the larvae of the amphibian *Xenopus laevis* due to short light exposure has been described by Dulcis and Spitzer (2008). In humans, Aumann et al. (2016) performed the first evidence of a dopamine switch in midbrain neurons and found that the density of TH+ neurons in the midbrain was higher in the brains of people who died in summer than those who did in winter.

The following are some other apparently general features of activity-dependent neurotransmitter switching in the studies:

- When describing a new neurotransmitter phenotype, the authors regularly verified that the observed changes in the number of DANs are not the result of neurogenesis or apoptosis but rather a change in the phenotype in existing neurons (Aumann et al., 2013; Tomas et al., 2015; Dulcis et al., 2013; Dulcis and Spitzer, 2008).
- The neurotransmitters that are the subject of the switch in the given experiments show an inverse relationship: the number of neurons with the new transmitter increases/decreases to the same extent as the number of neurons with the original neurotransmitter decreases/increases (Dulcis et al., 2013; Spitzer, 2017).
- In the case of this switch, it is probably a *de novo* induction of mRNA in specific transmitter systems (Dulcis et al., 2013). In our opinion, this finding prevents the idea that neurotransmitter switching is an extreme form of co-transmission, although it is possible that both these phenomena may occur in neurons parallelly.
- A change in transmitter identity leads to a change in postsynaptic receptors. Thus, ‘transmitter matching’ exists (matching of presynaptic neurotransmitters with postsynaptic receptors) (Dulcis et al., 2013; Dulcis and Spitzer, 2008; Spitzer and Borodinsky, 2008; Hammond-Weinberger et al., 2020).
- The change of a neurotransmitter in each neuron does not occur independently of the surrounding network. Gumez-Gamboa et al. (2014) described that a neurotransmitter switch is not cell-autonomous but depends on the level of activity in the surrounding neurons. Baker et al. (1983) described the loss of dopamine in neurons due to the loss of their afferentation (lesions of olfactory receptor neurons were associated with a dramatic decrease in dopamine levels and TH activity in the rodent olfactory bulb). We have already mentioned the neurotransmitter switch effect on the postsynaptic receptor expression.
- Neurotransmitter switching occurs in response to chronic changes in neuronal activity or chronic environmental changes; it is not an acute phenomenon (Spitzer, 2017). For the thesis of the flexible response of neurons by changing the dopamine phenotype, the speed of the switch is important. Aumann et al. (2011), in dopamine SNpc neurons of mice, describe the first increase in TH mRNA and in protein expression between 12 and 14 h. Conversely, Yang et al. (2002) describe a switch within 15 min in cell culture (15 min of brain-derived neurotrophic factor perfusion caused a switch from acetylcholine to norepinephrine in sympathetic neuron-myocyte connections in rodent cell culture).
- Changes in the dopamine phenotype can be dramatic: in culture of fetal rat sensory petrosal ganglion neurons, applying depolarizing stimuli to the environment led to an increase in the number of TH+ positive cells from 10% to 20–100% (Hertzberg et al., 1995).
- Changes in neurotransmitters are associated with changes in behavioural activity. Behavioural activity either induces a neurotransmitter change (for example, the dopamine phenotype grows the most in the learning phase of the movements that brings the benefit, (Aumann et al., 2013), or the change in the transmitter results in a

change in behaviour. Dulcis et al. (2017) describe that in larvae amphibians *Xenopus*, protracted exposure to kin odorants during development leads to increased numbers of DAN (and decreased GABA neurones) in accessory olfactory bulb interneurons – these changes in dopamine/GABA neuron ratio led to changes in attractiveness/avoidance in relation to kinship.

We presume that there is evidence of a relatively flexible change in dopamine equipment due to neuronal activity (laboratory-induced electrical activity, Ca2 +, and ion changes) or environmental changes. Furthermore, the number of DANs in the hypothalamus and midbrain of mammals (all vertebrates) can be regulated by environmental conditions. These changes occur by up- or down-regulation of the expression of genes and proteins for dopamine synthesis and do not respond to acute stimuli but to more chronic changes in the environment (as a change in network setting?). The relationship between reward functions and dopamine phenotype switching will certainly be an early subject of research, but we assume that longer-term changes in reward stimuli could lead to changes in the dopamine phenotype of neurons in the VTA.

2.5. Co-transmission and neurotransmitter switching in *Drosophila*

We mentioned in the previous paragraphs the possible capacity of dopaminergic areas of the brain and dopamine: 1) to occur and cooperate in co-transmission with other neurotransmitters, especially in reward functions, 2) to change the neurotransmitter identity flexibly, and express a particular transmitter according to the homeostatic and functional network needs. In our opinion, we continue to proceed from the idea that the principles of neuronal organisation and function are fundamentally similar throughout the animal kingdom.

We have described the dopaminergic parts of the *Drosophila* MB (i.e. the parts to which the reward functions are attributed) as relatively static and hardwired. Do DAN in *Drosophila* MB have a dynamic capacity similar to that described in mammals?

Several studies have been conducted on co-transmission in the *Drosophila* brain. Neuropeptides in *Drosophila* neurons modulate the function of small molecule neurotransmitters (fast-acting neurotransmitters and monoamines) often (Nassel, 2018). In the area of our interest, in MB, Barnstedt et al. (2016) identified the co-transmission of acetylcholine and short neuropeptide F (sNPF) in KC-sNPF enhances acetylcholine-evoked responses in MBON. A thorough overview of the co-transmission of two neuropeptides or a neuropeptide and a small molecule neurotransmitter was provided by Nassel (2018), but for the MB region, he did not report a different co-transmission than the mentioned Ach + sNPF in KC. In the first part of a single-cell transcriptomic analysis in *Drosophila* midbrain, Croset et al. (2018) stated that ‘certain neuropeptides preferentially accompany particular fast-acting transmitters, or monoamines’. Moreover, they describe the co-transmission of neuropeptides in DAN and state that up to 21% of DANs express the neuropeptides Dh44, Nplp1, glycoprotein hormone beta 5, and proctolin. Additionally at least one neuropeptide can be found in 61% of DANs and two or more of them can be found in 32% of dopaminergic cells. Interestingly, other monoamine neurons

(octopamine- and tyramine-neurons) have mRNA for many neuropeptides. Octopamine neurons: 85% of neurons express at least one neuropeptide, 46% express two or more Tyramine neurons: 83% express one neuropeptide, 78% express two or more. See Fig. 4.

Croset et al. (2018) also described the extent of monoamine co-transmission and fast-acting neurotransmitters. DANs express markers of the presence of fast-acting neurotransmitters (Vglut, Gad1, and VachT), although in a relatively small percentage, the percentage of octopamine and tyramine neurons with co-transmission is higher (see Fig. 5). The most striking result is that many octopaminergic and tyramineric neurons are likely to co-release glutamate. Let us clarify this by Spitzer findings (2017) that in laboratory studies, we often consider specific neurotransmitters from tests obtained under constant conditions.

Takemura et al. (2017) also contributed to the possibility of co-transmission in DAN, especially in the section describing presynaptic vesicles in DAN. They noted at least two morphologically distinct types of these vesicles and mentioned unspecified co-transmission as a possible explanation for the different presynaptic vesicles. They also noted the relationship between different presynaptic vesicles in the DAN and the innervation of specific MB compartments. Deng et al. (2019) tested the gene expression of all possible neurotransmitters and modulators and their receptors in *Drosophila* and concluded that co-transmission in the *Drosophila* brain is generally the norm (‘each transmitter/modulator/neuropeptide coexists with another, though in different regions and with different combinations’), and they clearly delineated against Dale’s principle. To increase the importance of the complex of these relationships between the transmitters and modulators for understanding the whole network, exceeding the importance of the description of a simple anatomical connectome, they formulated the concept of a chemoconnectome (‘as the entire set of neurotransmitters, neuromodulators, neuropeptides, and their receptors underlying chemo-transmission in an animal’). In this work, they paid particular attention to which neurotransmitters and neuromodulators occur in

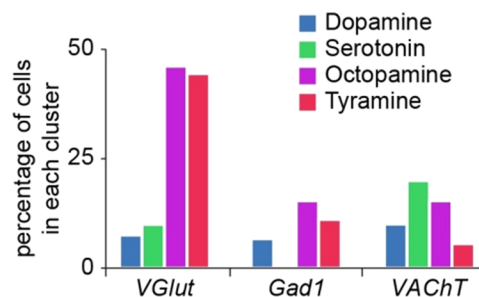


Fig. 5. : Illustration of the rate of co-expression of glutamate, GABA and acetylcholine in monoaminergic cells in *Drosophila* midbrain according to Croset et al. (2018). The columns show the percentage of cells in each monoaminergic cluster (dopamine, octopamine, serotonin, tyramine) that are co-expressing markers for co-release of glutamate, GABA, or acetylcholine. VGLut - vesicular glutamate transporter, Gad1 - glutamic acid decarboxylase 1, VACHT - vesicular acetylcholine transporter.

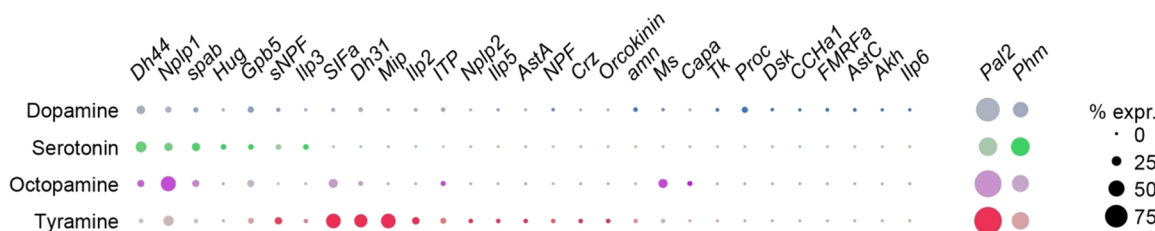


Fig. 4. : Figure after Croset et al. (2018) shows expression of genes encoding neuropeptides and neuropeptide amidating enzymes across monoaminergic populations in the *Drosophila* midbrain.

*Drosophila* DAN and described that 16 transmitters and neuropeptides might coexist with dopamine.

Research into the functional relationships of co-transmission in *Drosophila*, especially the search for the effect of co-transmission on behaviour, has only been carried out in recent years. Root et al. (2011) described the effect of sNPF co-transmission in olfactory neurons on increased food search behaviour, and Kapan et al. (2012) described the effect of neuropeptide co-transmission in sNPF-expressing neurons (dorsal lateral peptidergic neurons) on the regulation of stress resistance and metabolism. Further, Ignell et al. (2009) studied the colocalization of GABA and *Drosophila* tachykinin in local neurons in the antennal lobe and their effect on olfactory receptor sensitivity neurons with an indication of independent function. Further, above mentioned Barnstedt et al. (2016) characterized the effect of sNPF co-transmission in KC on the facilitation of the acetylcholine signal. From the point of view of our topic (co-transmission in DAN), Sherer et al. (2020) found that most octopamine neurons (about 70%) in the adult *Drosophila* brain also express the vesicular neurotransmitter transporter for glutamate (dVGluT).

The important question is whether co-transmission in the *Drosophila* brain is constant or it changes with the surrounding conditions. It is sex-dependent (Castellanos et al., 2013) and age-dependent (Davie et al., 2018); *Drosophila* aging is associated with 'regulatory erosion' (i.e. with a decrease in RNA content and with an increase in transcriptional and chromatin noise); however, the details are unknown.

Studies on the influence of EE on changes in brain structures have yielded interesting results. In general, the environment causes many types of neuronal changes in higher animals and has a more complex effect on brain plasticity (van Praag et al., 2000; Donlea and Shaw, 2009; Eckert and Abraham, 2013; Sale et al., 2014). The influence of EE on the expression of genes involved in neuronal structure and synaptic signalling has been described (Rampon et al., 2000). Furthermore, the role of epigenetic mechanisms in mediating the influence of EE on gene expression, especially transcription factors (Fischer et al., 2007; Sweatt, 2009) and the effect of EE on TH gene expression in mammals (Richard et al., 1988; Schalling et al., 1989). Similar results of the effects of EE on the *Drosophila* brain have been reported. The negative effect of the environment, especially during development, on the quality of brain structures is not surprising (Heisenberg et al., 1995; Wang et al., 2007; 2017). However, what would enrich our knowledge base in a better manner is the impact of positive EE on restructuring the damaged brain structures after removing the negative environmental cues. In 1984, Technau (1984) described an increase in the number of axons in the adult *Drosophila* MB due to EE (their EE focused on social contacts/isolation and sensory inspiration/deprivation), but further research will be needed in *Drosophila*. Ultimately, the whole topic of the influence of the environment on the neurotransmitter switching in an adult organism is an uncharted territory.

The mechanisms of transcriptional control of neurotransmitter identity in *Drosophila* have been studied (Thor and Thomas, 1997; Lacin et al., 2019), but it is too early to conclude anything comprehensively, and we must be satisfied at present with only indications of the neurotransmitter switching possibility in *Drosophila*.

Lacin et al. (2019) studied a method for creating the neurotransmitter identity of ventral nerve cord neurons in *Drosophila* and confirmed that this identity (acetylcholine, GABA, or glutamate) is obtained at the stem cell level but found that the acetylcholine-specific gene is transcribed in many neurons with different neurotransmitter identities (glutamate or GABA). However, these transcripts do not leave the nucleus and are not transcribed into proteins. In the discussion, however, they admit that this result could indicate neurotransmitter switching (under specific conditions such as a certain developmental stage or stress).

### 3. Discussion

In the review, we reflected on the unambiguousness of the neurotransmitter identity of DAN, which are key parts of reward networks in the mammalian and *Drosophila* brains (involved in the 'wanting' component of reward, arousal increasing, focusing attention on a pleasant/unpleasant situation, or reward prediction error). Neurotransmitter identity is not only due to the presence of a specific neurotransmitter in the cell but also by the presence of all elements of the signalling pathway of this transmitter (most of these studies are also based on the detection of TH, dopamine transporter, mRNA TH, or dopamine receptors). Current information suggests that the dopamine signalling pathway has similar biochemical and molecular elements in all animals, and DAN shares similar functional principles.

The parallel between the reward systems of mammals and insects is generally accepted (this parallel is based on the common dopaminergic identity of the neurons involved) (Perry and Barron, 2013; Waddell, 2013; Dvořáček and Kodrčík, 2021; Scaplen and Kaun, 2016; Cognigni et al., 2018); however, at the moment of relativization of neuronal identity due to the flexibility of neurotransmitter representations, the parallel might lose its meaning. What can make the difference between the dopamine neurons in the VTA and MB? What are the differences in this identity? Another rate of co-transmission? Neurotransmitter plasticity. We believe that theoretically, several possible answers and hypotheses are offered for further verification.

- 1) Theoretically, it is necessary to admit that both reward systems (mammalian and *Drosophila*) cannot be compared to each other to the extent that we do.
- 2) The research on DAN in mammalian VTA is more extensive than the research on DAN in *Drosophila*; in the future, the information will be supplemented and, on the contrary, the parallel will be confirmed.
- 3) Different flexibilities in neurotransmitter identity correspond to different reward functions. Interestingly, no fast-acting neurotransmitters have been found in the DAN of *Drosophila* MB. Why has the fast-acting component not yet been described in the *Drosophila* reward brain network? (We still assume that only the DAN is responsible for the reward signal). Is the rapid response to appetitive/aversive signals managed in another way? Without the need for reward labelling, for example, in the form of a labelled line (Scaplen and Kaun, 2016; Heimbeck et al., 2001; Busto, Cervantes-Sandoval and Davis, 2010), and only longer-term situations with reward potential require a change in the excitability setting by the slow-acting neurotransmitter (dopamine?)

Or is the interaction between two neurotransmitters (faster and slower) only required for reward prediction error? Do only mammals have the ability to evaluate the potential of reward as a higher evolutionary function of reward? There is some evidence of a reward prediction error in insects (Felsenberg et al., 2017; Terao, Matsumoto and Mizunami, 2014; Mizunami et al., 2017); however, these conclusions may be one of the possible variants of interpretation of the results.

- 4) Differences can be caused by the quality of the surrounding network, that is, the identity of a neuron can be given more by a place in the network than by a specific neurotransmitter. Glutamate in the DAN of mammalian VTA may not be primarily related to the dopaminergic nature of the neurons (i.e. with the specific neurochemical identity of the neuron, in our case with dopamine) but with the target network to which the neurons are connected (dual VTA DAN are connected with PFCs and medial shell NAc). *Drosophila* does not have these other integration networks - is this why they do not have a fast-acting transmitter expressed in DAN? It would be interesting to study DAN in cell culture, for example, from medial shell NAc cells, to determine whether the glutamate phenotype would not be expressed. We emphasise the idea that the transmitter identity can be strongly influenced by the target output network. The target network

may prompt DAN to express other genes than another network would, for example, co-transmission of other neurotransmitters or dynamic neurotransmitter switching.

We can consider the neuron as a universal element that is able under certain conditions and at a certain moment (development windows?) to express anything, depending on its location in the network. If we accept the idea that mammalian DANs express glutamate due to their connection to other (higher?) network sites, the question is: how does the neuron know the higher site of the network? Due to the information quality of work in this higher network part? Because of the simple fact of comprehensive information integration? Due to the sum of all the excitements in the network? Is a neuron, that finds itself in a larger information whirl, triggered to have higher transcriptional activity? Will this information quality trigger other parts of the network to express elements that higher network sites need?

- 5) A neuron in a mammalian reward network may represent a different organisational level than that in a *Drosophila* reward network. We tend to understand networks as a complex of neuronal connections - neurons are an essential element of this network; two information lines interact typically when they meet at a synapse. However, if there are regions on one neuron with different receptor variants or with different neurotransmitters, then the neuron itself can be perceived as a network itself. We hypothesise that in one animal, the integration of specific information may occur at the level of the neuronal junction, but in another, the same integration may occur at the junction of intraneuronal signalling pathways.

Neurotransmitter switching can be seen in vertebrates as a flexible tool for maintaining homeostasis to balance the rate of excitation/inhibition of a neuron at a given level of the network (Sale et al., 2014; Vogels et al., 2011). In *Drosophila*, this type of maintaining excitability homeostasis may not be so much needed - either its reward network does not need it because their reward signal is not so complex, or they replace this type of homeostasis in 'a network of one neuron' with another mechanism (e.g. the presence of multiple dopamine receptors on one cell with different influence on excitability). This strengthening of the network importance of one neuron in insects (*Drosophila*) can be evidenced, for example, by the peculiarity that some important functions stand on a single specific neuron, not on a group of neurons (Takemura et al., 2017; Wu et al., 2011; Lin et al., 2014).

- 6) The different results may be due to different conditions. We speculate that the repeated description of the exact anatomical and neurochemical identity of *Drosophila* DAN and, conversely, the dynamic identity of dopamine in mammals cannot be related to the ability to maintain much more constant conditions in *Drosophila* research than in mammals (e.g. pure genetic lines, easier maintenance of constant environment in short-lived organisms, less needs attributed to laboratory *Drosophila* than to mammals, etc.). Maintaining constant laboratory conditions to achieve the greatest possible comparability with other research may not be a suitable environment for studying neurotransmitter switching, especially if this switching is a reaction for longer-term changes only in the environment (moreover, any change in the environment made by the researcher may not be significant for *Drosophila*).
- 7) It must also be acknowledged that a similar 'neurotransmitter-centric' method of thinking about the brain may not be correct. For example, divergence and convergence occur in the action of neuromodulators, and the same action can be achieved by multiple modulators and vice versa (Brezina, 2010). Furthermore, the possibility of dramatically different effects of neuromodulators depending on a specific set of network parameters has been described (Gutierrez and Marder, 2014), and the same results can be achieved in a network using multiple solutions (Marder, 2011). Moreover, the role of dopamine in one part of the network may be different from that in another part of the network and is fundamentally different from its

role in the network of other animal species. Possibly, to understand a complex system such as the brain, it is necessary to consider the activity of individual network elements rather than the tool that caused the activity (the tool may be different in each network for each organism and relatively insignificant, i.e. sometimes expressed according to ad hoc needs). A general explanation of the possible function of dopamine, summarising the existing sub-concepts, was offered by Berke (2018): dopamine modulates resource allocation decisions, that is, it has a different function in each network. Thus, the resources are different in each part of the network. In the reward network (in the motivational part of the striatum), this resource is the animal's time (movement is the resource in the motor striatum; the cognitive process including attention in the cognitive striatum). If the animal finds the reward simply and in a straight line, then according to Berke (2018), it does not need the mesolimbic dopamine. Conversely, its role is needed if the organism has to render some temporally extended, strategically challenging activities.

However, despite these doubts, the topic of possible neurotransmitter plasticity in reward functions, including neurotransmitter switching in *Drosophila*, seems to be very attractive for further investigation. This could help address the hypothetical issues mentioned above.

#### 4. Conclusions

- 1) There is an obvious parallel relationship between the function of DAN in the reward systems of mammals and *Drosophila*, although the complexity of the function is probably different. There is also evidence that the dopamine signalling pathway has similar biochemical and molecular elements, and shares similar functional principles in all animals.
- 2) Co-transmission in dopaminergic neurons in mammalian VTA is documented as functionally necessary - dual neurons in the VTA form a specific subpopulation. In *Drosophila*, there is an evidence of co-transmission of DAN in MB (with neuropeptides or fast-acting neurotransmitters). However, a clear subpopulation of these dual neurons has not yet been identified, although there is evidence of a possible relationship between the co-transmission and output to specific MBONs.
- 3) The number of DANs, specifically, the number of cells with a dopamine phenotype, is activity-dependent in certain brain parts of certain vertebrates under certain conditions and may vary according to environmental conditions. In *Drosophila*, the possibilities of neurotransmitter switching are only indicated; studies focused on a possible switch of neurotransmitters in DAN will have to be conducted.
- 4) DANs have a solid capacity to contradict Dale's principle. The question arises if there is a neuron at all with one transmitter only in all circumstances that always resists environmental pressure and network activity to express genes other than those expressed during individual development.
- 5) The possible different capacity of mammalian and insect DAN (in our case *Drosophila*) to transmitter plasticity may be the cause (or consequence) of a different function of the reward system.

#### Contributions

The authors contributed equally to all aspects of the article.

#### Ethics declarations

The authors declare no competing interests.

## Acknowledgements

This study was supported by the project RVO 60077344 of the Institute of Entomology, Biology Centre, Czech Academy of Sciences, Czech Republic. A.B. acknowledges financial support from the Institute of Entomology, Biology Centre, Czech Academy of Sciences, Czech Republic, no. 620/8659. N.K. acknowledges financial support from Office of Research and Economic Development, Mississippi State University.

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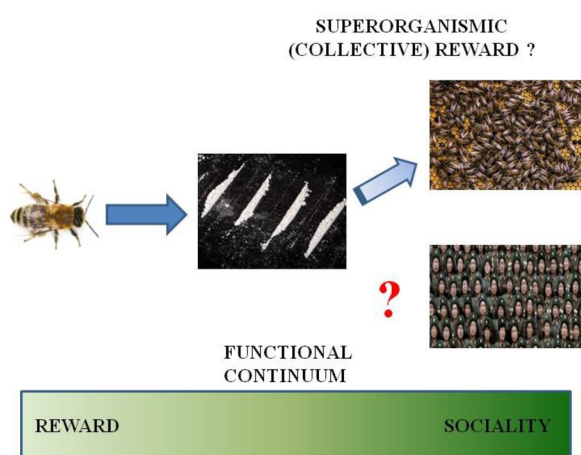
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# 5. KAPITOLA

## Článek 3 - Výzkum závislosti a efektů drog u hmyzu – od *Drosophily* ke kolektivní odměně u včely



Dvořáček, J., & Kodrík, D. (2022). Drug effect and addiction research with insects - From *Drosophila* to collective reward in honeybees. *Neuroscience and Biobehavioral Reviews* **140**, 104816. <https://doi.org/10.1016/j.neubiorev.2022.104816>





Contents lists available at ScienceDirect

## Neuroscience and Biobehavioral Reviews

journal homepage: [www.elsevier.com/locate/neubiorev](http://www.elsevier.com/locate/neubiorev)

Review article

Drug effect and addiction research with insects – From *Drosophila* to collective reward in honeybeesJiří Dvořáček<sup>a,b,\*</sup>, Dalibor Kodrík<sup>a,b</sup><sup>a</sup> Institute of Entomology, Biology Centre, Czech Academy of Sciences, Branišovská 31, 370 05, České Budějovice, Czech Republic<sup>b</sup> Faculty of Science, University of South Bohemia, Branišovská 31, 370 05, České Budějovice, Czech Republic

## ARTICLE INFO

## Keywords:

Brain reward system  
 Insect brain  
 Insect model of addiction  
 Addiction  
 Collective reward  
 Honeybee brain  
*Drosophila* brain

## ABSTRACT

Animals and humans share similar reactions to the effects of addictive substances, including those of their brain networks to drugs. Our review focuses on simple invertebrate models, particularly the honeybee (*Apis mellifera*), and on the effects of drugs on bee behaviour and brain functions. The drug effects in bees are very similar to those described in humans. Furthermore, the honeybee community is a superorganism in which many collective functions outperform the simple sum of individual functions. The distribution of reward functions in this superorganism is unique - although sublimated at the individual level, community reward functions are of higher quality. This phenomenon of collective reward may be extrapolated to other animal species living in close and strictly organised societies, i.e. humans. The relationship between sociality and reward, based on use of similar parts of the neural network (social decision-making network in mammals, mushroom body in bees), suggests a functional continuum of reward and sociality in animals.

## 1. Introduction

The consequences of substance abuse pose extremely high health, social, and economic burdens. In studies examining the global burden of disease, disorders associated with the use of alcohol, tobacco, and other addictive substances have been the top-ranked health risks for many years. Indeed, the 2019 Global Burden of Diseases, Injuries, and Risk Factors Study reported that alcohol use was the leading risk factor for adults aged 25–49 years (GBD, 2019 Risk Factors Collaborators, 2020).

Understanding the principles of drug addiction is complicated, not least because of the indescribable complexity of the human brain. Using animal models (meaning mammals) in addiction research can partially reduce the complexity of the problem, but insect models enable researchers to significantly simplify the complexity of brain networks when examining the effects of drugs.

Addiction is a social construct that refers to a number of distinct states, each of which manifests itself differently in various people and has shifting symptoms over time. Many current addiction theories correspond to this heterogeneity of addiction. A thorough summary of addiction theories, including an attempt at a synthetic theory of addiction, is provided by West and Brown (2013). According to these authors, addiction as 'a syndrome at the centre of which is a repeated powerful

motivation to engage in a rewarding behaviour, acquired as a result of engaging in that behaviour, that has significant potential for unintended harm'. Addiction is thought to be a dysregulation of the brain reward system on a neurobiological level (Koob and Le Moal, 2001), which is originally a concept derived from the study of the mammalian brain. Its basic function is to mark biologically important stimuli (events, particularly related to food and mating, important for the survival of an organism) with a hedonic value to increase the probability of the organism seeking this biologically important stimulus. When addictive substances are consumed, the parameters of the reward system continually shift up to a state of addiction (Everitt et al., 2008; Kourrich et al., 2015; Koob and Le Moal, 2008). The current neurobiological model of addiction with clinical and public health consequences is clearly described, for example, in the work of Volkow, Koob and McLellan (2016). Presently, the presence of the reward system in the brains of lower organisms, such as insects, has been well-documented, and similar principles of brain reward systems in humans, mammals, and insects are similar (Perry and Barron, 2013; Waddell, 2013; Dvořáček and Kodrík, 2021). However, studying the effects of drugs on the mammal/human brain is complicated not only by the complexity of the brain, but also by the presence of different levels of understanding of brain activities, including neurobiological (neurophysiological) and psychological levels. Both levels use

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<https://doi.org/10.1016/j.neubiorev.2022.104816>

Received 8 April 2022; Received in revised form 29 July 2022; Accepted 1 August 2022

Available online 5 August 2022

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incompatible concepts; neurobiological concepts are not usually able to explain brain functions at the level of ordinary human experience, which can be achieved using concepts at the psychological level. However, precisely defining brain function at the psychological level is difficult since the terms are often old and ineffectively describe the principles of brain networks. Employing invertebrate animals in neurobiological research is attractive not only for the simplicity and relative clarity of their brain functions, but also enables using terms from only one level of understanding of brain activity (neurobiological). The general principles of operation can then be abstracted by comparing differently organised nervous systems. In this case, the general principles underlying the action of drugs on neural networks and adaptation of these networks to chronic action can be extrapolated from insects to humans (Kaun et al., 2012; Ryvkin et al., 2018; Devineni and Heberlein, 2009; Søvik and Barron, 2013). The ambition of addiction neuroscience is, among other things, to identify principles of the brain-drug relationship that are not anthropomorphic. Søvik and Barron (2013) pointed out that the very definition of addiction according to the American Psychiatric Association (Diagnostic and Statistical Manual of Mental Disorders, APA, 2013) is significantly anthropomorphic and criteria for addiction must be adjusted for non-human models. Thus, insect models can help clarify the essential features of addiction.

Currently, two insect models are mainly used in neurobiological research - the fruit fly (*Drosophila melanogaster*) and honeybee (*Apis mellifera*). The genomes of both organisms have been sequenced (Adams et al., 2000; Honeybee Genome Sequencing Consortium, 2006). Despite many similarities, *Drosophila* and honeybees differ in brain complexity. In *Drosophila*, the basic neurophysiological principles of reward functions are more readable, whereas the brain network of the bee is several levels more complex, but remains relatively clear. The presence of higher forms of learning and a substantial social component of reward makes the honeybee a more attractive model for extrapolation to humans.

In this review, we focus on honey bee as an insect model to study the reward functions and the impacts of drugs as factors damaging the reward system. We compare the bee reward system with a more common insect model, *Drosophila*, used in neurobiology, and highlight certain traits that could promote the honeybee as a relatively more interesting model for comparison with the mammalian/human brain. Presently, studies on the effects of drugs on bees are still limited; thus, this lack in relevant literature could promote several novel ideas and concepts to study the mammalian/human reward system.

Reward functions involve several components. Based on the classification of reward functions according to Berridge (2008, 2009), in this review, we have used the following components: 'wanting' (motivational drive, desire to obtain a reward), 'liking' (main hedonic component, pleasure itself), and 'learning' (in the form of classic and instrumental associations and cognitive representations of the reward).

## 2. *Drosophila* reward network as a basis for other insect models

### 2.1. Associative learning and reward system in *Drosophila*

The reward system of the *Drosophila* brain is studied regularly, with research predominantly investigating olfactory learning and conditioning by appetitive (mostly sweet sugar) or aversive stimuli (e.g. quinine or electric shock) (e.g. reviewed in Lowenstein and Velazquez-Ulloa, 2018). The basic parameters of the reward system in *Drosophila* have been elucidated, including its similarity with reward systems of higher animals (Perry and Barron, 2013; Waddell, 2013; Scaplen and Kaun, 2016; Dvořáček and Kodrál, 2021).

The basic network for the formation of olfactory associations under the influence of reward in *Drosophila* is composed of thousands of neurons. One mushroom body contains approximately 2200 key Kenyon cells (KCs), 130 important dopaminergic neurons for reward, 34 output neurons (Aso et al., 2014), and hundreds of thousands of synapses

(Takemura et al., 2017), which are organised into four basic levels: connection of olfactory sensory neurons and projection neurons in antennal lobe, connection of projection neurons from antennal lobe and KCs in calyx mushroom body, connection of KCs with mushroom body output neurons (MBONs), and connection of dopamine neurons (DANs) or octopaminergic neurons with MBONs (Schwaerzel et al., 2003; Claridge-Chang et al., 2009; Aso et al., 2010; Waddell, 2010; Oswald and Waddell, 2015; Yamagata et al., 2015; Cognigni, Felsenberg and Waddell, 2018). Association formation is considered to occur where information converges about conditioned (originally neutral stimulus, here odour) and unconditioned (reward stimulus, e.g. sweet sugar) stimuli. The KC-MBON-DAN synapse is predominantly understood to be this location in *Drosophila*. Although the reward mushroom body is a key area in the main model, some authors emphasize the role of the lateral horn. For example, Galizia (2014) demonstrated the primary role of the mushroom body in odour identification, but suggested that attributing appetite/aversive value to a particular odour and motivational status selection takes place in the lateral horn.

This network is highly structured internally, with several types of individual cells and asymmetric connections within the network likely originating during different developmental ages (Dvořáček and Kodrál, 2021). Nevertheless, apart from the internal irregularities and asymmetries of the structure, a simplified model of this network is probably sufficient for quality associative learning with reward, which has also been investigated using computational modelling of these networks (Patanè et al., 2018; Mosqueiro and Huerta, 2014; Gupta et al., 2018). The benefit of these biologically-inspired spiking neural networks is, among other things, that they contain the basic parameters essential for successful operation of the network, i.e., a three-layered network (antennal lobe layer, KC layer, mushroom body layer) + lateral horn + reward value source (Smith et al., 2008; Huerta and Nowotny, 2009).

### 2.2. Inhibitory recurrent connections in the associative network of *Drosophila*

The associative network of *Drosophila* is not as feed-forward as the simplified model might suggest, but contains many feedback loops, recurrent connections, and two special neurons, anterior paired lateral neurons (APLs) and dorsal paired medial neurons (DPMs), which form key recurrent connections. APLs form the GABAergic recurrent loop for maintenance of sparse coding (Lei et al., 2013; Lin et al., 2014), while DPMs form recurrent loops important for memory consolidation (Cervantes-Sandoval and Davis, 2012; Keene et al., 2004, 2006). APLs and DPMs can also form a self-sustained feedback loop (APL-DPM-some parts of KC), where reverberation activity in the network can lengthen the memory trace and lead to improved stabilisation (Wu et al., 2011; Pitman et al., 2011).

### 2.3. *Drosophila* and reward without nutritional value

Most studies investigating the *Drosophila* reward system have been conducted using an appetite stimulus with nutritional value (sugar), which can be misleading for insect models of drug action or addiction. For example, sugar attractiveness can be controlled by physiological mechanisms other than pleasure per se. Drugs as artificial sources of mammalian reward (Everitt et al., 2008; Kourrich, et al., 2015; Koob and Le Moal, 2008) raise the question of whether insects are also attracted to drugs only because of their pleasure value, without any other additional biological importance, or even in spite of negative parameters (e.g. toxicity, unpleasant taste, or competition for other natural rewards).

*Drosophila* appears to dominantly prefer stimuli with nutritional value (Burke and Waddell, 2011), which is regulated by the fly's metabolic status (satiety/hunger) and is therefore not absolute (Krashes et al., 2009). Several studies have described the attraction of *Drosophila* to drugs even in the presence of aversive stimuli. In these experiments, flies were attracted to alcohol at the cost of electric shock (Kaun et al.,

2011) or the presence of bitter quinine (Devineni and Heberlein, 2009). Although the idea that *Drosophila* is attracted to the same drugs as humans/mammals is generally agreed upon (Kaun et al., 2012; Landayan and Wolf, 2015; Heberlein et al., 2009), the mechanisms underlying this attraction remain unclear. For example, endogenous opioids and endocannabinoids, which mediate drug attractiveness in mammals, are absent in *Drosophila* (Mirabeau and Joly, 2013; Jékely, 2013; Elphick, 2012).

### 3. Associative learning and reward system in honeybees

#### 3.1. Anatomical basis for honeybee associative learning

Associative learning in bees has been well documented (Eisenhardt, 2014; Tedjakumala and Giurfa, 2013; Paoli and Galizia, 2021; Søvik et al., 2015). The reward system of the bee has similar principles of organisation as that of *Drosophila*, but differs in some important respects that make this insect model more interesting for human neuroscience. The bee brain is significantly more complex than that of *Drosophila*. The number of cells in the bee brain is estimated at 1000,000 (Witthöft, 1967), which differs significantly from that of *Drosophila* (approximately 100,000 neurons) (Raji and Potter, 2021), indicating a different functional burden in the main brain center of associative learning for the bee. The mushroom body of *Drosophila* contains approximately 2500 cells compared to 300,000 cells in the mushroom body of the bee, accounting for 2% of brain volume in *Drosophila* compared to 20% in bees (Menzel, 2012).

The basic neuroanatomical substrate for associative olfactory learning in bees is essentially the same as that in *Drosophila*, including a synapse of sensory and projection neurons in antennal lobe glomeruli, a synapse of projection neurons and KCs in the mushroom body calyx, simultaneous direction of projection neurons into the lateral horn, a system of feedback neurons, and output from the mushroom body and lateral horn to the next/effector neuropils (Paoli and Galizia, 2021; Zwaka et al., 2018; Mobbs, 1982). However, the complexity of networks at all levels in the bee is an order of magnitude higher than that in *Drosophila*.

The first network that processes olfactory stimulus in the bee is the antennal lobe, which is functionally composed of 163 glomeruli. A glomerulus is a micro-network where olfactory sensory neurons with the same receptor converge and simultaneously meet a large number of local interneurons (approximately 4,000) and projection neurons (approximately 800), which differ by the simplicity/complexity of the input and target area of the projection (mushroom body/lateral horn) (Zwaka et al., 2018; Paoli and Galizia, 2021; Witthöft, 1967). Associative learning and formation of some forms of memory in bees are processed at this level (Farooqui et al., 2003; Giurfa, 2003).

Another part of the network where sensory information is processed is the mushroom body. This is likely the main region where sensory stimuli are processed, reward value is evaluated, and the behavioural response is formed. Associative learning already occurs at the mushroom body calyx level (Haenicke et al., 2018). Two calyces on each side of the bee brain process multisensory information (olfactory, visual, mechanosensory, and gustatory), unlike in *Drosophila* where only dominant olfactory information is processed at the calyx level (Strausfeld, 2002; Mobbs, 1982). In the bee calyx, axons of projection neurons form synapses with KCs, of which there are approximately 184000 (versus 2200 in the mushroom body of *Drosophila*). Interestingly, drones have a higher number of neurons and fewer KCs than worker bees (Mobbs, 1982). KCs in bees are not a homogeneous group of cells, but have at least two types that differ macroscopically, form different network connections, and have different functional roles (Strausfeld, 2002). Synapses of projection neurons and KCs, together with feedback GABA neuron terminals and octopaminergic/dopaminergic neuron terminals, form functional microcircuits that are important for reward processing (Paoli and Galizia, 2021). In contrast to *Drosophila*, where the output

area from KCs is the most important region of association formation with valence reward evaluation (synapse KC-MBON-DAN, e.g. Schwaerzel et al., 2003; Claridge-Chang et al., 2009; Aso et al., 2010; Waddell, 2010), association formation has been most studied at the calyx level in bees (Hourcade et al., 2010; Haenicke et al., 2018; Szyszka et al., 2008).

#### 3.2. Feedback system in honeybees

A special feature of the bee mushroom body network is the highly developed feedback system. KCs are connected with mushroom body extrinsic neurons, including 110 GABAergic A3 neurons, half of which are feedback neurons, which connect mushroom body output regions (lobes) with input regions (calyx) (Grünewald, 1999; Zwaka et al., 2018; Haehnel and Menzel, 2012). The robustness of this system is apparent when compared to the single feedback GABAergic APL neuron in *Drosophila* (Lin et al., 2014). The importance of this strong feedback system for associative learning is crucial (Raccuglia and Mueller, 2014; Zwaka et al., 2019), although not entirely clear. GABA feedback can be important for solving ambiguous tasks (Boitard et al., 2015), combining multiple sensory modality (Zwaka et al., 2019), modulating input depending on experience (Filla and Menzel, 2015), mediating reinforcement of unconditioned stimulus (Raccuglia and Mueller, 2014) and is likely essential for maintaining sparseness of KC activity (Froese, Szyszka and Menzel, 2014; Szyszka et al., 2005). Grünewald (1999) assigned feedback neurons the role of a secondary integration center for connecting non-corresponding parts of the mushroom body. In addition to the overall higher number of cells in the mushroom body of the bee compared to that in *Drosophila*, the main neuroanatomical difference is the feedback system.

#### 3.3. Higher quality network, higher quality learning

Bee associative learning occurs at multiple levels, including the antennal lobe and mushroom body. The antennal lobe is probably sufficient for elemental learning, while the mushroom body is likely required for non-elemental forms of learning (Giurfa, 2003). However, evidence of plasticity during learning of odours at the antennal lobe level has also been observed in *Drosophila* (Thum et al., 2007). In general, associative plasticity may not be limited to these two network sites (antennal lobe, mushroom body), but may be a function freely distributed throughout the network, with each neuron responding to associative learning to varying degrees (Okada et al., 2007). Thus, creating memory at the antennal lobe level may not be restricted to bees, it has only been more studied in this insect model.

In many aspects, the higher-quality network (i.e. more neurons with more feedbacks) in bees corresponds with better learning compared to *Drosophila*. Not only can a bee learn more characteristics of a potential food source at the same time, including the temporal and spatial context of that source in a multisensory manner (Eisenhardt, 2014; Filla and Menzel, 2015; Zwaka et al., 2019), the bee is also capable of more complex (non-elemental) forms of learning (Giurfa, 2003; Perry, Barron and Chittka, 2017). Giurfa (2003) suggested that higher forms of bee learning show similar features to vertebrate learning (contextual learning, categorization, and learning abstract rules), labelling them cognitive functions in which cognition was defined as "the sum of processes by which information is processed, stored, retrieved and used in a flexible and adaptive sense". Devaud et al. (2015) also described "higher-order cognitive abilities" in this context. Several studies have described higher-order cognitive operations in honeybees that otherwise occur in vertebrates. The bee is able to learn stimulus categories, generalize stimuli, create basic stimulus categories in memory, and use them according to context (Menzel, 2012). In addition, the bee is capable of concept learning, i.e., the ability to learn the relationship between two stimuli and create basic concepts such as "sameness/difference" (Avarguès-Weber and Giurfa, 2013). Attention-like processes are also documented in bees, not only behaviourally, but

with evidence of brain correlates (Paulk et al., 2014; Perry et al., 2017). Vertebrate-like specialities of learning also include social learning, defined as the ability of a group to learn from an individual's experience (Perry et al., 2017). According to Barron and Klein (2016), the bee's ability to perceive space and its own position and movement within it, combined with the bee's capacity for selective attention in visual fixation of an object (i.e., the ability to create environmental representations subjectively and "egocentrically") provides evidence of the bee's capacity for subjective experience as an evolutionary basis for (later) development of consciousness.

#### 4. Superorganism and collective reward

##### 4.1. New form of community – the superorganism

The consequences of the reward system of the bee must be considered in the context of one essential feature of this insect model. The bee does not form an ordinary community as a simple group of many individuals, albeit hierarchical and centrally controlled. The bee colony behaves in many ways as an independent organism, creating an evolutionarily new type of community – a superorganism (Bernadou and Kramer, 2021; Moritz and Fuchs, 1998; Canciani et al., 2019). Working and reproductive roles are strictly divided into individual castes, which differ predominantly in their approach to the outside/inner world in relation to food acquisition/storage/consumption (with food considered a natural source of reward), reproduction (again emphasizing the aspect of natural reward in other animals), degree of dependence on other colony members, and levels of associative learning (Canciani et al., 2019; Alaux et al., 2010; Johnson and Frost, 2012). Strict reproductive differentiation (sterile workers) creates the conditions for the colony to function as a high-level unit, where the colony's profit dominates the individual's profit without conflict between individual and colony interests (Bernadou et al., 2021; Moritz and Fuchs, 1998). The performance and skills of the colony are higher and better than those of individual bees or even the sum of individual bee contributions.

Collective learning and decision performance in the colony are of higher quality than those of individual bees (Sasaki and Pratt, 2018). Moreover, the colony copes with environmental stress (i.e., superorganism resilience) much better than the individual bee (the number of genes associated with detoxification and immunity is reduced compared to the genome of solitary insect species) (Straub et al., 2015; Berenbaum and Liao, 2019). Another example of this phenomenon is colony versus individual thermoregulatory ability (Grodzicki et al., 2020). In addition, the colony's working abilities are flexible. The bee colony can respond quickly to changing environmental conditions by altering the structure of its castes (Johnson and Frost, 2012) and/or by modifying biochemical and physiological attributes (Kodrík et al., 2022).

##### 4.2. Distribution of reward functions

Managing a community as highly organized as a superorganism likely relies on a combination of self-organization, local decision-making, and hierarchical second-order signals, especially pheromones (Moritz and Fuchs, 1998; Alaux et al., 2010; Canciani et al., 2019). Reward-related functions are unevenly distributed in the colony, with each caste and worker group participating in a defined part of the reward behavior of the entire colony. Associative learning, which is predominantly used to find and evaluate food sources, is probably best developed in foragers because their activities are affected by several fluctuating factors, including their own number as the total work capacity of all foragers, quantity and quality of nectar and pollen supply, and storage filling in the nest (Seeley, 1986). Evaluating the quantity and quality of food is apparently performed by nurse bees (Crailsheim, 1990).

Foragers have well-developed associative learning, reflected by the significantly increased size of the mushroom body calyx with more KC

dendrites and pruning PN axons than other bees (Muenz et al., 2015), but their activity does not lead to individual reward. The effect of individual activity is not evaluated since colony profit is most important, thus the individual probably does not know and/or need to know this information (Moritz and Fuchs, 1998; Johnson and Frost, 2012). In nurse bees, the quality of learning is kept intentionally low by queen mandibular pheromone (Beggs et al., 2007).

The queen, who influences the reward setting of the whole colony, for example by her queen mandibular pheromone levels (Alaux et al., 2010; Fischer and Grozinger, 2008), probably does not follow the achievement of reward, but rather specific parameters of the colony structure, such as worker density (Alaux et al., 2010; Canciani et al., 2019; Smith et al., 2017). The queen's possible reward functions, such as state of satiety, remain unknown. However, the queen probably lacks the more developed associative learning that is a condition of reward functions, judging by her relatively small mushroom body compared to that of worker bees (Roat and da Cruz Landim, 2008). The only theoretical candidate for preserved individual reward functions remains the drone, whose role is less dependent on other members of the colony. For example, unlike worker bees, aversive associative learning in drones remains unaffected by social alarm pheromones (Avalos et al., 2017).

##### 4.3. Collective reward?

Although the bee provides a suitable insect model for studying reward functions, the bee in the colony has likely undergone individual reward sublimation. Therefore, it will be more effective to talk about superorganismic reward (collective reward) and to study it at the colony level. This is probably the general principle underlying the distribution of basic functions in the colony as mentioned above (Berenbaum and Liao, 2019; Straub et al., 2015; Grodzicki et al., 2020). Thus, individual reward-directed behavior can be replaced by a new form of social organization (Søvik et al., 2015).

#### 5. Complex roles of biogenic amines

##### 5.1. Biogenic amines in *Drosophila* reward

Biogenic amines, especially dopamine and octopamine, play important roles in associative learning and reward. Dopamine has traditionally played a key role in the reward theories of various animals (O'Connell and Hofmann, 2011; Barron, et al., 2010; Ikemoto et al., 1997; Schultz, 1997; Spanagel and Weiss, 1999; Burke et al., 2012; Blomberg-Martin et al., 2010). The main hedonic transmitter is usually considered to be dopamine, but there are more explanations for the role of dopamine in reward functions and associative learning (Dvořáček and Kodrík, 2021; Berridge, 2008). The dichotomous role of biogenic amines in associative learning was originally established in *Drosophila*, as the first insect model of reward functions, with dopamine having an aversive value and octopamine having an appetitive value (Schwaerzel et al., 2003; Riemensperger et al., 2005). Later experiments indicated that octopamine only played a role in the formation of short-term memory after ingestion of sweet sugar (Waddell, 2010) and the dichotomy of the role shifted to different DAN groups (Mao and Davis, 2009; Aso et al., 2012; Claridge-Chang et al., 2009). Currently, the roles that specific DANs play in the reward valence (positive/negative) are considered to be more flexible and universal. Most DANs are probably involved in both aversive and associative learning, or this valence depends on many other parameters (Handler et al., 2019; Yamagata et al., 2016). In any case, a dopaminergic system is probably needed to mediate the attractiveness of a drug in *Drosophila* (Freyberg et al., 2016; Bainton et al., 2000; Kaun et al., 2011).

It is entirely possible that the role of dopamine in reward functions (and thus, for example, in mediating the effects of drugs) will expand beyond this basic role. The original role of dopamine in reward functions in both mammals and insects has already been replaced by much more

complex significance. In reward functions, dopamine was crucial for the ‘wanting’ component [the complicated role of dopamine in reward functions has been discussed comprehensively in a review by Dvořáček and Kodrík (2021)]. In our context, in addition to the ‘wanting’ component, many studies have reported the dominant role of dopamine while focusing on an interesting or new stimulus or labelling the novelty of the stimulus (Blomberg-Martin et al., 2010; Schultz et al., 1993). Dopamine causes a decrease in the attention threshold (arousal threshold) for a given environmental stimulus (Kume et al., 2005; Crocker et al., 2010), and associative learning is then directed in the desired direction (Krashes et al., 2009). In general, dopamine regulates everything worthy of attention, but it can also selectively increase attention depending on where in the network it is located (Blomberg-Martin et al., 2010; Fiorillo et al., 2013). Attention focusing and pleasure drive, defined as ‘wanting’ by Berridge (2008), may not be so different. In principle, attention focusing and pleasure drive may be a single function or a continuum manifested differently in different situations. After all, a much smaller specialization of brain functions is generally most likely (Anderson, 2010).

### 5.2. Biogenic amines in bee learning

Biogenic amines play different roles in bee reward than in *Drosophila*. In bees, octopamine is essential for appetitive learning (Hammer, 1993; Hammer and Menzel, 1998; Vergoz et al., 2007a), while dopamine function is key for aversive learning (Vergoz et al., 2007a). The important role octopamine plays in bee reward functions is based on the octopaminergic ventral unpaired median neurons of the maxillary neuromer 1 (VUMmx1), their connection with the antennal lobe, mushroom body, and lateral horn (Hammer, 1993), and subsequent experiments investigating associative learning with sucrose within the Proboscis extension response paradigm (Hammer and Menzel, 1998). However, Søvik et al. (2015) pointed out that the morphologically similar (and with similar network connections) octopaminergic neuron of *Drosophila* (VUMa2) does not have this function in reward and different methodologies (genetic/pharmacological methods) were employed to study the roles of biogenic amines in *Drosophila* and bees. Thus, these authors expressed doubts that octopamine/dopamine antagonists used in previous experiments were sufficiently receptor specific to reliably distinguish the functions of biogenic amines in bees. However, the role of dopamine in bee associative learning is fixed today: dopamine conveys an aversive value to unconditional stimuli (Tedjakumala et al., 2017; Wright et al., 2010).

The roles that biogenic amines play in bee reward are likely to be more ambiguous and less straightforward than those in *Drosophila*. Nevertheless these roles are likely to be diminished in favour of a broader neurochemical continuum (Shohat-Ophir et al., 2012; Wright et al., 2010), which is flexible according to the needs of the bee and current environment (review of possible neurotransmitter switching and cotransmission in the *Drosophila* reward system Dvořáček et al., 2022).

### 5.3. Social aspect of biogenic amines

In bees, biogenic amines also affect the social structure and relationships within the bee community. Bees of a similar age with different roles in the hive differ significantly in biogenic amine composition. Food storers have significantly lower dopamine levels than comb builders, whereas hive defenders have significantly lower octopamine levels than foragers (Wagener-Hulme et al., 1999). The two key amines have opposite effects in the community. Dopamine exerted a strong effect on bee sociability, likelihood of interaction with other bees, and nestmate affiliation, while octopamine reduced social interactions with other bees (Hewlett et al., 2018). Dopamine was also reportedly involved in the defensive behaviour of bee guards (Novvian et al., 2018). The results of this study are interesting for comparative considerations about the relationship among social stimulus (sting alarm

pheromone), increased dopamine (and serotonin) levels, probable impact on attention focusing, and increased probability of an attack with an altruistic aspect (self-sacrifice of stinging bees).

Biogenic amines are also likely to mediate pheromone effects on the community. Queen mandibular pheromone, which blocks aversive learning in young bees (Vergoz et al., 2007b), altered dopamine levels, gene expression of dopamine receptors, and the cellular response to dopamine (Beggs et al., 2007).

If biogenic amines are related to associative learning and reward, as well as to social roles and relationships within the bee colony, a two-way effect is possible. The social position of a bee in the colony may affect the perception of reward and conversely, experience with reward can change the bee’s position in the colony. Thus, reward associations and roles in the community can form a continuum.

## 6. Honeybees and alcohol

In the study of the effect of alcohol on bees, three main questions arise: 1. Can alcohol have a hedonic value that is sought after by bees? 2. Is the acute effect of alcohol on bees similar to that on mammals? 3. Do chronic doses of alcohol induce similar effects as in mammals, i.e. signs of addiction?

### 6.1. Attractiveness of alcohol

The hedonic value of alcohol for bees remains unclear. In humans, the reasons for alcohol use are psychological or social, which are difficult to prove in insect models. The anxiolytic/anti-stress effects of alcohol may be explored in neurophysiological research, but studies with insect models are unknown. Attraction to alcohol by insects can thus be evidenced by voluntary search and use, or preference over another substance. Bees were unlikely to have an aversion to low-alcohol solutions (Abramson et al., 2004a, 2004b), and were able to regularly consume 1%, 5%, 10%, and 20% alcohol solutions (although some defects occurred with increasing concentration, such as negative locomotory effects) and 95% alcohol with certain reservation (Abramson et al., 2000). Bees preferred alcohol over sucrose solution (Ostapchec et al., 2021; Abramson et al., 2004a) or in the form of sweeter alcoholic beverages commonly produced for human consumption (Abramson et al., 2004a), but not in water (Mustard et al., 2019). Possibly, the aversive taste of alcohol was masked by sugar. However, according to Varnon et al. (2018), conditioned taste aversion to alcohol was not produced in bees.

Sokolowski et al. (2012) documented free-flying foragers searching for 5% alcohol solution (with 50% sugar), despite alcohol consumption causing disadvantages such as smaller size of pollen load. These authors also noted large differences in the responses of individual bees (some bees did not respond to alcohol, while the opposite behaviour was recorded for others). Satiety was not observed in this experiment - the bees worked throughout the period of alcohol consumption.

Self-administration of alcohol by bees and preference over a sugar solution in a 2-feeder choice experiment was documented by Mustard et al. (2019). Alcohol preference was dose-dependent; bees preferred 1.25% and 2.5% solutions, but did not avoid 5 or 10% solutions. Alcohol tolerability may be related to the normal presence of small amounts of alcohol in food sources, but preference over sugar by bees is an interesting result considering the negative acute effects of alcohol, aversion to antennae contact with alcohol, and absence of nutritional value of alcohol (Mustard et al., 2019).

### 6.2. Acute alcohol intoxication

According to the Diagnostic and Statistical Manual of Mental Disorders, alcohol intoxication in humans manifests as inappropriate sexual or aggressive behaviour, mood lability, impaired judgment, and at least one of the following: slurred speech, incoordination, unsteady gait,

nystagmus, attention or memory impairment, stupor, or coma (APA, 2013). The signs of acute alcohol intoxication in bees are similar:

- impaired cognitive function, including significantly impaired classical proboscis extension reflex conditioning (Abramson et al., 2000, 2004a, 2004b, 2005; Maze et al., 2006), significantly impaired quality of associative learning, acquisition and strength of association, and impaired attention levels (Mustard et al., 2008), and reversal of learning (Abramson et al., 2015).
- increased aggressiveness of free-flying bees (Abramson et al., 2004a, 2004b). Notably, the authors of one experiment described one of the few disadvantages of the bee as a model organism: "the...bees breached the laboratory and began stinging people inside the building."
- reduced work performance with smaller size of food load brought back to the hive and longer intervals between flights (Sokolowski et al., 2012) or prolonged return time of foragers with food to the hive (Bozic et al., 2006),
- disrupted motor function, including reduced locomotion in shuttle box and running-wheel tests (Abramson et al., 2000), reduced walking time in a Petri dish, prolonged breaks in movement, complex changes in grooming and flying (Maze et al., 2006), loss of coordination, and gradual disorientation to sedation (Ammons and Hunt, 2008). In free-flying foragers, return time to the hive was increased and the number of such returns decreased with increasing alcohol dose (Stephenson et al., 2021). Effects on motor skills were dose-dependent as in mammals (Maze et al., 2006; Ammons and Hunt, 2008).
- dose-dependent attenuation, sedation (Miler et al., 2021), and subsequent death (Maze et al., 2006).
- negative effects on social behaviour, including reduced waggle dancing and disrupted food exchange (the main forms of bee communication) (Bozic et al., 2006; Mixson et al., 2010). The social impacts undoubtedly vary according to castes and specific responsibilities. Acute alcohol intake has a major impact on the structure of the bee community. In one study, administration of 10% alcohol to the queen bee caused a delay in egg laying, and although she was accepted by the workers after being returned to the hive, the previously intoxicated queen was replaced by a new queen two weeks later (Cakmak et al., 2009). Bozic et al. (2006) also noted that alcohol-intoxicated foragers spent more time self-cleaning.
- Further, signs of an excitatory phase of alcohol intoxication, similar to that known in humans, were noted in one experiment in which bees consuming 5% alcohol exhibited increased walking behaviour 40 min after alcohol administration (Maze et al., 2006). It remains unclear if these effects occur at the expense of other roles in the community.

### 6.3. Chronic effects of alcohol and addiction

Investigating the long-term effects of alcohol and possible development of addiction in animals/insects is difficult using current medical criteria. Only two of the signs of addiction are physiological in nature and verifiable in animal models - development of tolerance and presence of withdrawal (Søvik and Barron, 2013). Other features are mostly psychological, but may be partly documented behaviourally, such as impaired control during alcohol use, continued use despite the presence of adverse negative effects, preference over other activities/sources of reward, and intense desire to use alcohol most likely in an environment that was previously associated with alcohol consumption (APA, 2013).

As mentioned above, bees display a preference for alcohol over sugar solution and continued use despite the presence of negative effects. Moreover, several studies have provided evidence of adaptation mechanisms to chronic alcohol use in bees. Miler et al. (2018) described the development of tolerance to alcohol-induced motor damage, observing that the negative effects of ethanol on motor function were mitigated in

bees that received concentrated alcohol 5 days before the experiment. Stephenson et al. (2021) tested the effect of alcohol on the working functions of free-flying foragers (including returning to the hive), reporting that bees accustomed to alcohol were able to maintain their working functions after administration of alcohol in contrast to bees that had not developed an alcohol tolerance. A recent study documented bee withdrawal from alcohol (Ostap-Chec et al., 2021). The authors used increasing alcohol concentrations in sugar solution and demonstrated withdrawal from discontinued use associated with an increase in mortality. However, this group of "addicted" bees rejected ethanol in water, which undermined the idea of craving aimed at raising the alcohol level in the body in any form.

## 7. Bees and non-alcoholic drugs

### 7.1. Cocaine as a representative stimulant drug

Unlike alcohol, bees do not come into contact with cocaine naturally under circumstances that could attract them. Cocaine does not signal the presence of a food source, on the contrary, cocaine has insecticidal properties that result from a potentiating of insect octopaminergic transmission (Nathanson et al., 1993). Nevertheless, as is demonstrated in everyday life, the toxicity of a substance does not protect against its voluntary use. Cocaine is sought after for its psychostimulatory and centrally analeptic and anorectic effects (APA, 2013). However, the attractiveness of these effects is linked to human life context and societal values (e.g. high performance, slenderness), which is difficult to imagine for insects. Nevertheless, Søvik et al. (2014) confirmed that bees developed a preference for the location where they received cocaine.

The psychostimulatory effects of cocaine on bees, better described here as cognitive- and behavioural-stimulating effects, are parallel to those on humans. Acute low doses of cocaine improved foraging activity (reflected by increased source visits), increased proboscis extension response for sugar and sting extension response for electric shock (Søvik et al., 2014), and caused a dose-dependent increase in the probability and rate of waggle dancing after return to the nest (Barron et al., 2009). At the same time, these authors described the absence of other motor malfunction or hyperactivity in isolated bees, which they interpreted as evidence of the effect of cocaine not directly on motor skills, but primarily on reward assessment and processing, specifically resulting in overestimation of food source value. Similarly, Søvik et al. (2014) reported that the increased foraging activity after cocaine use was mainly related to the low-quality sugar solution, suggesting that cocaine changed the bee's perception to overestimate the sugar concentration. These results offer a parallel to human exaggeration of self-confidence, grandiosity, and megalomania with cocaine use. Incidentally, a similar effect on increased reporting of resource value has been documented for octopamine (Barron et al., 2007).

The same research laboratory (Søvik et al., 2018) tested the effect of acute doses of cocaine on memory function, reporting a strong inhibitory effect on memory extinction and demonstrating that cocaine acts on memory directly by changing DNA methylation dynamics and not through the process of incentive salience. However, the stimulating effects on dance behaviour were most likely caused by the interaction of cocaine with biogenic amines since the administration of a biogenic amine receptor (mianserin) ended the above-mentioned effects on dance behaviour (Barron et al., 2009). In contrast, other experiments (Søvik, Cornish and Barron, 2013) have not demonstrated changes in brain biogenic amine levels associated with altered bee motor skills after acute and chronic cocaine use.

Bees display possible addiction-like adaptive changes due to chronic cocaine. Barron et al. (2009) documented the presence of a withdrawal-like state in bees after cessation of regular cocaine administration, manifested as a deterioration in learning compared to bees with continued chronic cocaine use. Further, Søvik et al. (2013) documented the development of physiological tolerance to cocaine in motor

function. Repeated administration of low doses of cocaine led to less effect on motor function during subsequent high doses of cocaine. The phenomena of place-preference (Søvik et al., 2014), development of tolerance (Søvik et al., 2013), and withdrawal-like status (Barron et al., 2009) suggest the addictive potential of cocaine for bees, despite the reason for the attractiveness of stimulant drugs for invertebrates remaining unclear.

### 7.2. Heroin as a representative sedative drug

Little is known about the effects of heroin or other opioids on bees. An early study by Núñez et al. (1983) explored morphine suppression of the stinging response to electric shock and whether this effect was attenuated by naloxone. In this study, morphine clearly inhibited the stinging response to stress, while naloxone attenuated the effect, suggesting that bees must have opioid receptors. The endogenous opioid system in mammals is considered to be a key factor in mediating a hedonic component reward (Berridge and Kringelbach, 2015), but it remains unclear whether this 'liking' component can be mediated by another system, except the endocannabinoid system (Mitchell et al., 2018). However, components of an opioid system have not been found in the bee genome (Weinstock et al., 2006) nor in that of *Drosophila* (Mirabeau and Joly, 2013; Jékely, 2013), indicating that insects likely do not have an endogenous opioid system as it is known in mammals. Nevertheless, similar effects might be mediated by a system of neuropeptides. Indeed, 36 neuropeptide-encoding genes, including 9 unique genes associated with neuropeptides, have been identified in the bee genome (Weinstock et al., 2006). The same conclusion applies to endocannabinoids; a relevant endogenous cannabinoid system has not been identified in insects (Elphick, 2012).

Regardless of the absence of an endogenous opioid system, opioids have effects on bees. As expected, acute administration of morphine to bees suppressed locomotive activity, disrupted acquisition of short- and long-term associative memory, and slightly impaired long-term memory consolidation especially associated with aversion (Fu et al., 2013). Another study (Hassanpour-Ezatti, 2015) explored the effect of heroin on the sucrose responsiveness threshold (measured by proboscis extension reaction) and locomotive activity of bees, reporting different effects depending on the dose; very low heroin doses stimulated activity, while higher doses suppressed activity.

## 8. Superorganism and drug action

### 8.1. Drug effects on superorganism

If the bee colony is considered a superorganism while also considering the hypothesis of collective reward, interesting results of the effects of drugs on the entire superorganism could be observed. Unfortunately, research in this area is still in its nascent stages. Nevertheless, some effects of drug exposure on bee colonies have been reported. In one study, the alcohol-intoxicated queen was accepted back into the hive, but she laid fewer eggs and was subsequently expelled (Cakmak et al., 2009). Alcohol exposure also disrupted several types of social behaviour in foragers (Bozic et al., 2006). The aforementioned impaired communication (Mixon et al., 2010; Bozic et al., 2006), work performance (Stephenson et al., 2021; Sokolowski et al., 2012; Bozic et al., 2006), and increased mortality (Maze et al., 2006) certainly have an impact on the colony.

Higher (toxic) doses of drugs will presumably evoke the same defense mechanisms as the colony applies in response to other pollutants or stress, including termination of intoxicated individuals (Straub et al., 2015). However, at lower, non-toxic drug doses, long-term addiction adaptations of the entire colony/superorganism may occur as they do in multicellular organisms.

## 9. Conclusions

Using insect models to study the effects of drugs and all aspects of the reward system is attractive for several reasons. The two most widely used insect models - *Drosophila* and the honeybee - share common neurobiological principles of reward function, but differ in the following important aspects: neuroanatomy (the bee has a more complex brain network - 1000,000 neurons versus 100,000 neurons in *Drosophila*), sensory signal processing (the mushroom body of the bee is multi-modal compared to the uni-modal, dominantly olfactory signal processing in *Drosophila*), and complexity of the feedback system (much more complex in the mushroom body of the bee than in that of *Drosophila*). The neuroanatomical conditions of the bee likely enable more complex forms of learning, which are essentially similar to vertebrate learning.

The bee's complex learning capacity (or basal cognitive functions) makes it an interesting insect model for human neuroscience. Where neurophysiological principles of reward processes are not readable due to the excessive complexity of human brain functions, the bee offers a relatively simple model with the basics of higher brain functions and provides an attractive model for comparative studies of addiction. Thanks to bee social learning and sociality, studying the effects of drug use on an individual's behaviour in the context of their social group is possible with bees.

Both insect models have advantages. The bee model offers greater complexity (often easier extrapolation to vertebrates), but as Søvik and Barron (2013) point out, the genetic tools in the range and precision used for *Drosophila* are not yet available for bees. Performing experiments in parallel using the same methodology for both insects would be informative. *Drosophila*, as a simpler, more readable model, can offer results in the form of basic neurophysiological principles. Meanwhile, the same experiment conducted with complex bees, having higher brain functions and highly socially conditioned brain activities, can offer an additional system level of understanding while remaining sufficiently readable.

When researching human brain activity, the distinction between neurophysiological principle and psychological level is complicated or even impossible. Parallel research with *Drosophila*-bee models and suitably chosen vertebrate-human models can offer interesting extrapolations and comparisons. For example, such parallel research could assist in defining the general principles of reward or identifying the neurophysiological core of specific parameters/manifestations of reward and identifying a psychological superstructure (in the case of addiction treatment, this differentiation of the nature of problems could result in more accurate pharmacotherapy/psychotherapy). Addiction is a complex phenomenon that must be understood from a variety of perspectives, including the dysregulation of the brain reward system. Addiction has a tendency to alter every component of the more complex motivation system (West and Brown, 2013). Our work focuses on the central component of the motivational system, which is likely to be shared by all animals throughout evolutionary history — albeit in varied forms. All attempts to develop comprehensive theories of addiction can be supported by the universality of reward functions and the universality of these networks' responses to addictive drugs. With these facts in mind we can consider whether the uniqueness of a reward function found in specific animal species is an exception or a general principle that has not yet been explored in this context.

In exploring the social consequences of drug use, the bee colony - a superorganism - provides extraordinary insight. Combining the individual (superorganism as one entity) and social (colony as a community of many individuals) aspects of the bee colony generates new questions about the relationship between individual and collective rewards. Are individual reward functions, even those that are highly developed (such as the forager's complex learning ability), deprived in the superorganism? Is individual reward in the organized community sublimated and replaced by a higher-level, collective reward? Undoubtedly, the basic principles of the social consequences of drug action can be studied using

the bee model, but studying the effect of drugs on a superorganism as a single entity will provide interesting information.

The relationship between sociality and reward is an interesting question. O'Connell and Hofmann (2010) compared the evolution of the mesolimbic dopaminergic (reward) system and social behaviour network in various groups of vertebrates (mammals, birds, reptiles, amphibians, and teleost fish), suggesting the two closely linked networks are one "social decision-making network." This integrated network connects reward and social behaviour functions, thus creating a functional continuum. In this context, the mushroom body plays a crucial role in the neural correlation of social behaviour in the bee as a structure connected with reward functions (Paffhausen et al., 2020: mushroom body extrinsic neurons are important for controlling social interactions). Insect sociality probably does not affect the size of the mushroom body, but a large mushroom body due to demands on associative learning and complex orientation in space may be a pre-adaptation for social development (Farris, 2016). The above-mentioned parallel with the mammalian brain and similar evidence of neuroanatomical proximity of reward and sociality suggest a broader functional system, taking the form of reward functions in some species but parameters of social behaviour in others. The absence of individual reward in the bee superorganism supports this hypothesis, and collective reward could be a manifestation of complex social behaviour.

The relationship between individual and collective rewards or sublimation of individual reward in a strictly organised, complex community can have important philosophical consequences in attempting to extrapolate the findings to human communities. Is it possible to apply the experience of collective reward to human communities? Is it possible to deduce consequences for human society from the effect of drugs on a superorganism? Do human societies differ at different times due to changing degree of "superorgasmity" in the values attributed to individual or collective reward? Can a higher degree of sociality fully replace individual reward? Furthermore, a possible functional "sociality-reward" continuum, in which importance is assigned to reward for controlling organism behaviour, may be a small anthropocentric distortion in that it is judged by humans, i.e. a species with developed individual reward functions. Nevertheless, we believe that similar issues can, among other things, contribute to more effective therapeutic interventions and prevention strategies in the field of human addictology.

## Ethics declarations

The authors declare no competing interests.

## Acknowledgements

This study was supported by the project RVO 60077344 of the Institute of Entomology, Biology Centre, Czech Academy of Sciences, Czech Republic.

## Contributions

The authors contributed equally to all aspects of the article.

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# **6. KAPITOLA**

## **Závěr**

Hmyz má relativně dobře rozvinutý mozek a dobře rozvinuté mushroom body jako základní strukturu pro asociální učení a pro libostní funkce. Genom je u hlavních modelových druhů (*Drosophila melanogaster* a *Apis mellifera*) kompletně popsán. Současně jde o relativně jednoduché nervové sítě, které lze přehledně zkoumat – na rozdíl od mozku savců/člověka. Hmyzí modely samozřejmě již jsou hojně využívány v laboratorních experimentech. Základní principy fungování a organizace nervové sítě jsou univerzální napříč živočišnou říší a lze tedy úspěšně extrapolovat výsledky zkoumání mozku těchto jednoduchých modelových organizmů na zkoumání mozku člověka. Libostní funkce (systém odměny) patří mezi ty, u kterých je univerzalita a evoluční konzervativnost zřetelná. Studium libostního systému hmyzu může inspirovat či doplnit i teorie a hypotézy, které máme o poruchách libostního systému, zejména o závislosti. Užívání návykových látek přináší mimořádně vysokou zátěž zdravotní, sociální i ekonomickou, porozumění mechanismům závislosti je proto mimořádně důležité, problém je ale bohužel nahlížen mnoha často vzájemně nekompatibilními způsoby. Přesné porozumění (neurobiologické) podstatě závislosti přitom může pomoci nastavit nejen správné terapeutické intervence, ale i snižovat celkové zatížení společnosti důsledky závislosti. Srovnání informací z hmyzích modelů s informacemi ze studia působení drog na savce a s klinickými informacemi z lidské medicíny může pomoci objasnit základní neurofyziologické principy vzniku závislosti, společné znaky závislosti živočichů a jedinečné znaky člověka, naznačit evoluci jednotlivých úrovní libostních funkcí a pomoci formulovat méně antropocentrickou diagnózu závislosti. Bez podobných zjednodušení pravděpodobně nelze překonat současný složitý systém teorií a hypotéz o závislosti formulovaných z různých dílčích, někdy i zcela významem periferních hledisek. Samotná evoluce jednotlivých úrovní libosti a pravděpodobná paralelnost těchto evolučně různě starých funkcí v mozku vyšších živočichů dává zajímavé inspirace: jednak ukazuje, že problém závislosti není zdaleka jen psychologický či behaviorální, ale má fyziologické jádro spojené s udržováním homeostatického kontinua organismu, jednak naznačuje, že závislost může u různých jedinců být poruchou různých úrovní libostního systému (tedy pojem závislost může skrývat více typů závislosti) a že komplexní přístup v léčbě by měl tuto neurofyziologickou a neurochemickou složitost a mnohvrstevnost závislosti reflektovat.

Podobné komparace dvou skupin živočichů velmi od sebe vzdálených v evolučním stromu může být inspirativní pro další témata neurobiologie či celé fyziologie: jedinečné aspekty lidské fyziologie a jedinečný design struktury s tím spojené lze lépe pochopit komparací co nejširšího spektra živočichů. Paralelní výzkum čtyř modelů (octomilka-včela-savec-člověk) může nabízet zajímavé extrapolace a závěry.

Ze zpracovaných materiálů může plynout i několik dílčích inspirací pro další výzkum:

- V laboratorních experimentech zaměřených na systém libosti u nižších živočichů se často atraktivita konkrétního podnětu posuzuje podle zvýšené aktivity (např. motorické) či reaktivity (např. proboscis extension response u *Drosophily*). Toto má logiku u sledování funkcí podobných komponentě “wanting”, ale nelze tak zachytit případné “liking-like” funkce, které by měly vyvolávat ztlumení aktivity (pozn. nesdílíme ten názor, že zmiňovaný proboscis extension response je paralelou k faciální

expresi vyšších živočichů). Mohlo by být zajímavé nalézt takový design experimentu, který by mohl zachytit „satiety-like“ stav.

- Opioidová funkce “liking” může být završením evoluce komplexnější neuropeptidové homeostázu vyladující funkce (viz např. obrázek 4). V kombinaci s předpokládanou flexibilitou role neuropeptidů se nabízí zaměřit pozornost na vztah různých peptidů a různých aspektů “liking-like“ funkce.
- “Liking” funkce u nižších živočichů byla obecně málo dosud zkoumaná – buď byly experiment zaměřeny na roli (absentujících) endogenních opioidů nebo na zmiňované zvýšení aktivity modelového živočicha. Zkoumání případné “liking” funkce bude vyžadovat oprostít se od antropocentrické představy této funkce.
- K dalšímu zkoumání vybízí myšlenky kontinuity libosti a prostředí (např. Má prostředí a způsob života vliv na strukturu libostního systému?), kontinuity sociálnosti živočišného druhu a odměny (Jde opravdu o jeden širší funkční systém, který u některých druhů nabývá více tvaru sociálnosti a u jiných spíše libostních funkcí?) nebo vztahu mezi individuální a kolektivní odměnou u člověka.

Podobná zkoumání mohou mít i širší než biologický význam. Chápání univerzality libostních funkcí, tedy i přisouzení základní funkce libosti i vývojově jednodušším živočichům, může mít dopady i filozofické a ekologické. Lidé můžou mít větší respekt k živočichům, kterým jsme schopni přisoudit minimálně zárodky proto-emocí, kterými vnímání libého/nelibého bezesporu je.

## 7. Literatura

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