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Physiological and pathological processes of synaptic plasticity and memory in drug discovery: Do not forget the dose-response curve

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ABSTRACT

The response of a biological system to an endogenous or exogenous molecule depends upon the dose. For this reason, performing dose-response curves is crucial to understand physiological and pathophysiological phenomena, and to predict the effect of a drug. Most of the studies in pharmacological research have been performed according to the classical threshold model, focusing on higher doses able to ensure a biological effect. However, recent evidences pointed out the need to investigate the effect of low doses. Indeed, several molecules behave in a hormetic fashion, i.e. low-doses stimulate whereas high-doses inhibit a biological response. This is particularly interesting in CNS, where several physiological molecules involved in neuronal transmission during learning and memory have shown a biphasic effect that might represent the link between physiology and pathology.

In this review we will focus on cholinergic, glutamatergic and nitrinergic transmission, because of their central role in learning and memory and their impairment in neurodegenerative disorders such as Alzheimer's disease.

Pre-clinical studies performed on healthy adult animals and aged animals, as well as transgenic animal models of AD, have suggested a biphasic DR for acetylcholine, glutamate and nitric oxide. This stresses the relevance to perform DR curves when studying the mechanisms underlying synaptic plasticity and memory, the pharmacological profile of cognitive-enhancing drugs acting on these systems, and the possibility to combine low/ineffective doses of drugs that might have additive/synergistic effects, reducing the unwanted side effects associated to the high doses.

1. Introduction

Est modus in rebus. Sunt certi denique fines, Quos ultra citraque nequit consistere rectum (Q. Horatius Flaccus, 35 BC)

The concept that "the response depends upon the dose" is ancient and can be applied to various aspects of existence. From the "μηδὲν ἄγαν" (mēdén ágan = nothing in excess) carved into the Apollo Temple in Delphi, to the "Est modus in Rebus" (there is a proper measure in things) stated in Horatius Satire, the right amount has been constantly suggested to maintain the proper balance in life.

In medicine, hyper- and hypo- states lead to a malfunctioning of the body, whose homeostatic balance is ensured by the proper quantity.

In pharmacology, the relevance of the dose was already evidenced in the 16th century by Philippus Aureolus Theophrastus Bombastus von Hohenheim, probably more known as Paracelsus, who summarized the concept in his famous sentence: "Alle Dinge sind Gift, und nichts ist ohne Gift; allein die Dosis machts, daß ein Ding kein Gift sei" (all things are poisonous and nothing is without poison; only the dose makes a thing not poisonous), i.e. "Sola dosis facit venenum". This suggested the modern concept of dose-response (DR) describing the functional response of a biological system to different doses (in terms of exposure or concentration) of a compound.

Nowadays, we can rely on a wide variety of drugs and improving the selectivity and safety of compounds is crucial. Thus, DR studies are commonly applied in pharmacological research to standardize and foresee the expected response of a biological system at a given dose of a drug (Aronson et al., 2007).

These studies allow to interpolate a set of empirically gathered data points in a DR curve, which, in turn, is the graphical representation of a

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function. The latter can be used to extrapolate data and calculate several characteristic of a compound, i.e. its pharmacokinetic and pharmacodynamic profile (Standing, 2017).

For several decades, the most used approach to model a DR curve from empirical data was represented by the Hill model, firstly introduced by Hill to explain the relationship between oxygen tension and the saturation of hemoglobin (Hill, 1919). Since then, the same model has been used to estimate the required amount of a compound able to bind its receptor to produce a functional response (Goutelle et al., 2008). Although this approach might ensure an excellent model in many circumstances, it is limited by the fact that a variety of compounds exhibit a multi-phasic response, whereby a first order exponential decay curve is not sufficient to represent their DR curve. Consequently, the application of Hill model, which considers single-inflection DR curves represented by linear functions, can alter the data interpretation (Weiss, 1997). Accordingly, the interaction between different doses of a compound and the response of a biological system is more likely to change from a beneficial/adverse effect to the opposite effect when increasing the dose (Lushchak, 2014). This biphasic DR phenomenon characterized by low-dose stimulation and high-dose inhibition has been defined "hormesis" (Mattson, 2008). The corresponding graphical representation consists in a J-shaped or U-Shaped DR curve (Lushchak, 2014). The stimulatory and inhibitory effect should be present in a DR continuum and, more importantly, the hormetic stimulatory effect ends in correspondence of the so-called No-Observed-Adverse-Effect-Level (NOAEL), representing the starting point of a traditional DR curve (Dorato and Engelhardt, 2005). In fact, according to the classical threshold model, the NOAEL represents the level of exposure to a substance at which there are no biological effects, whereas the Lowest-Observed-Adverse-Effect-Level (LOAEL) represents the lowest dose at which there is an effect. These parameters, usually used in toxicology to calculate the "safe" concentration of risk agents or drugs not inducing adverse effect compared with control, might also be used to evaluate the beneficial effects of a drug. In the hormetic context, the NOAEL can be considered as a border delimiting two dose ranges, a stimulatory/inhibitory dose in the left side, and an opposite response range in the right side of the curve.

Unfortunately, pharmacological studies have obtained limited information on the DR below the NOAEL, especially because most of the toxicological studies were not designed to detect a possible biological effect below the "safe" threshold of no-adverse effect. On the other hand, the sensitivity of the measurements should increase together with the decrease of the dose (Davis and Svendsgaard, 1990).

Recently, a great effort has been made to validate the biological stimulatory effects hidden under the NOAEL. When screening more than 8000 compounds by performing a complete DR curve (reviewed in: Calabrese, 2008), it was revealed that the biphasic response is a common feature of both exogenous toxic substances and endogenous molecules.

This concept has represented a remarkable step towards the comprehension of several pathophysiological mechanisms and the development of new treatments, because the same molecule might exert a beneficial or a negative effect depending upon the dose (Calabrese, 2008).

Recently, attention has increasingly focused on the possible biphasic role of physiological molecules involved in neuronal transmission during learning and memory. Among these, here we will focus on: i) cholinergic transmission, for the central role of muscarinic and nicotinic receptors in learning and memory (Hasselmo, 2006); ii) glutamatergic transmission, for its involvement in long-term potentiation (LTP), a form of long-lasting synaptic plasticity due to a functional and structural modifications of synaptic strength that is thought to represent the cellular mechanisms underlying memory (Bliss and Collingridge, 1993); iii) nitrinergic transmission, for the peculiar function of nitric oxide (NO) as a retrograde messenger and the involvement of the NO/cGMP pathway in synaptic plasticity and memory (Lu et al., 1999). On the other hand, a dysregulation of these forms of neurotransmission has been recognized as a possible *primum movens* in diseases affecting cognition in the elderly as well as in patients with Alzheimer's disease (AD) (Mufson et al., 2008; Bollen and Prickaerts, 2012; Revett et al., 2013;), so that most of the therapeutical approaches rely on drugs acting on acetylcholine (Ach), glutamate or NO/cGMP pathway. Interestingly, recent studies have demonstrated that amyloid-beta peptide (Aβ), whose increase has been considered one of the main pathogenetic factors leading to the cognitive deficit in AD, might also act as a neuromodulator when at low physiological concentrations, boosting synaptic plasticity and memory (Puzzo et al., 2008, 2012).

Here we will discuss pre-clinical studies performed on healthy adult animals and aged animals, as well as transgenic animal models of AD, suggesting that these molecules behave in a hormetic fashion. The knowledge of these characteristics might be useful to better understand the physiology of learning and memory, as well as the pathophysiology of diseases characterized by their impairment. Moreover, it will contribute to detect the most efficacious minimum dose of cognitive enhancing and neuroprotective drugs, the therapeutic window to operate within, and how to combine low/ineffective doses of drugs that might have additive/synergistic effects, reducing the unwanted side effects associated to the high doses.

2. Cholinergic system

2.1. Physiological function in learning and memory

Ach is a neurotransmitter distributed in autonomic, peripheral and central nervous system. It acts as a classical excitatory neurotransmitter at the neuromuscular junction and in the autonomic ganglia, whereas in the brain it has been recognized as a neuromodulator able to modify neuronal excitability and presynaptic release of neurotransmitters, to induce synaptic plasticity and to coordinate the firing of groups of neurons in response to adequate stimuli (Picciotto et al., 2012).

Ach effects are mediated by pre- and postsynaptic receptors classified as metabotropic muscarinic receptors (mAChRs) and ionotropic nicotinic receptors (nAChRs) (Picciotto et al., 2000). mAchRs stimulation is able to induce both excitatory and inhibitory effects on the release of other neurotransmitters depending upon the subtypes involved. M1, M3 and M5 mAchRs subtypes are coupled with Gq proteins and lead to the activation of phospholipase C, whereas M2 and M4 subtypes are coupled with Gi/o proteins and lead to the inhibition of adenylyl cyclase. nAChRs, instead, function as cation channels generally leading to neuronal depolarization.

Despite the modality used by Ach to influence neuronal transmission is still a matter of debate, several evidences have suggested the primary role of the cholinergic system in learning and memory, attention and reward (Gold, 2003; Hasselmo, 2006).

Loss of cholinergic neurons in the basal forebrain, as well as progressive degeneration of the cholinergic innervation of the hippocampus and cerebral cortex, has been shown in AD patients (Delacourte and Defossez, 1986; Cummings and Benson, 1987). Furthermore, these patients also show a reduction in the activity of the Ach synthesizing enzyme choline acetyl transferase in these regions (Coyle et al., 1983; Davis et al., 1999; DeKosky et al., 2002). These findings probably contribute to the dysfunctional memory in patients with AD and have led to investigations into the involvement of cholinergic receptors for cognition enhancement (Toyohara and Hashimoto, 2010).

2.2. Cognitive-enhancing drugs acting on the cholinergic system

2.2.1. Acetylcholinesterase inhibitors

One of the first studies aimed to investigate whether Ach was involved in learning and memory was performed in the early '70s

(Deutsch, 1971). One of the previous studies aimed to inhibit the physiological breakdown of Ach by using acetylcholinesterase inhibitors (AChEIs), thereby increasing the level Ach in the synaptic cleft. Authors performed a DR curve on the effect of the AChEI physostigmine on a maze task. Interestingly, physostigmine-enhancing effect on cognition followed an inverted U-shaped function, with higher doses inducing a decline of performance (McGaugh and Petrinovich, 1965). This study paved the way for several clinical applications aimed to improve cognition by inhibiting Ach degradation (Christie et al., 1981; Beller et al., 1985; Mohs et al., 1985; Gustafson et al., 1987).

It is now accepted that AChEIs can produce modest improvements in cognition, but are not free of side effects due to the inhibition of acetylcholinesterase (AChE) in the periphery (Hansen et al., 2008). The occurrence of these side effects, consisting in nausea, vomiting, diarrhea, abdominal cramping and anorexia, is dose-dependent and this underlines the importance of being in the right spectrum of the DR curve, maintaining the minimum effective dose able to enhance cognition with the minimum side effects.

AChEIs are available in 'reversible' and 'irreversible' variants. Irreversible AChEIs form a permanent covalent bond with the enzyme AChE and via this way totally inhibit enzyme function. These compounds are used as insecticides in gardening and agriculture, and as chemical military weapons in lethal nerve gases. Compounds that reversibly inhibit the enzyme AChE are used clinically as the above described cognitive enhancers, delaying the onset of AD. The reversible AChEIs are less toxic and shorter acting and do not form permanent covalent bonds with AChE (Lanctôt et al., 2009).

The first reversible AChEI was physostigmine, derived from the calabar bean more than 100 years ago (Giacobini, 2000, 2003). Since then, several inhibitors have been proposed to maximize efficacy and minimize side effects (Giacobini, 2003).

At now, AChEIs approved by the FDA for the treatment AD are tacrine (Cognex), donepezil (Aricept), rivastigmine (Excelon), and galantamine (Reminyl) (Hansen et al., 2008; Lockhart et al., 2009), all behaving in a hormetic fashion with low doses stimulating cognition.

Unfortunately, the modest efficacy of these drugs combined with their side effect profile has limited the clinical usefulness (Lanctôt et al., 2009). For example, tacrine, the first approved AChEI, was taken off the market in the UK due to unforeseen liver toxicity when chronically administered to patients. This led to the development of second generation AChEIs (Lanctôt et al., 2009), among which galantamine and rivastigmine are nowadays indicated for mild-to-moderate dementia, whereas donepezil can be used in all phases of dementia.

Galantamine, an alkaloid obtained from Galanthus nivalis L., has been traditionally used for its cognitive-enhancing effects in popular medicine (Howes et al., 2003). Several clinical trials have demonstrated that it is well tolerated (Wilcock et al., 2000; Wilkinson and Murray, 2001) and might have therapeutic advantages compared with other AChEIs because it also stimulates nAchRs (Biton et al., 2007; Lanctôt et al., 2009).

Of all AChEIs, donepezil is most widely used in the AD and dementia population. Donepezil has an excellent oral bioavailability and easily crosses the blood-brain barrier. A slow and gradual increase in the dose of donepezil is an important factor for obtaining a balance between clinical efficacy and tolerability, thereby maximizing any positive effects on cognition while minimizing the incidence of negative side effects (Lockhart et al., 2009). Donepezil appears to be more selective for AChE in the brain than in the periphery, is not associated with liver toxicity (as tacrine) and produces fewer gastrointestinal side effects compared to other AChEIs (Hansen et al., 2008; Lockhart et al., 2009). These cholinergically-mediated gastrointestinal side effects are dose-dependent (Courtney et al., 2004), thus supporting the importance of using a dose located in the optimal spectrum of the DR curve, to avoid the increasing incidences of peripheral side-effects as well as the cognitive impairment due to high doses administration, as shown in preclinical studies with acute administration (e.g. Prickaerts et al., 2005).

Even if AChEs are not able to act as disease-modifying dugs, other studies have been performing to develop an ideal inhibitor that should quickly cross the blood brain barrier to reach the brain, but rapidly disappear from the periphery to minimize the adverse side effects (Giacobini, 2003).

2.2.2. mAchRs and nAchRs ligands

An additional strategy to improve cholinergic transmission is based on the stimulation of AChRs. Both mAchRs and nAchRs have been targeted to develop cognitive-enhancing drugs to be used in AD and other diseases characterized by cognitive impairment and behavioral disturbances.

mAchRs involved in cognition are the post-synaptic M1 subtype and the pre-synaptic M2 subtype. M4 and M5 subtypes are also distributed in the brain, but their function is still doubt, even if the M4 has been suggested to modulate dopaminergic signaling (Jeon et al., 2010). Agonists of M1 receptors have been demonstrated to improve cognition in pre-clinical studies, but, at now, they have not been approved for clinical use due to side effects and modest selectivity for M1 (reviewed in: Clader and Wang, 2005). For example, xanomeline, an M1/M4 agonist, has been proved to slightly ameliorate cognitive deficits in AD patients in a phase III clinical trial. However, its effect was more against behavioral disturbances, with a dose-dependent response (Bodick et al., 1997; Foster et al., 2014). For this reason, clinical trials are trying to unravel the potential therapeutic use of xanomeline as antipsychotic in diseases affecting behavior, such as schizophrenia (Shekhar et al., 2008), thanks to the M4-mediated antidopaminergic effect.

Several other M1 agonists, such as CI-1017, talsaclidine, WAY-132983, cevimeline, AC-42, LY-593093, CDD-102, have shown a potential cognitive-enhancing effect in animal models. However, they did not enter clinical trials due to a high-risk low-benefit profile.

Also antagonists of M2 receptors, which increase Ach levels by disinhibiting its release after neuronal firing, have been tried on preclinical models. They have shown an effect against behavioral and cognitive symptoms in AD. They displayed less peripheral gastric side effects, but tachycardia since brain and cardiac M2 receptors are identical (reviewed in: Clader et al., 2005; Foster et al., 2014).

Another strategy based on cholinergic transmission is stimulation of nAChRs, which has been demonstrated to have dose-dependent effects in the CNS. The main agonist of nAchRs, i.e. nicotine, has been demonstrated to delay the aging process of nigrostriatal neurons (Prasad et al., 1994). Furthermore, nicotine has been demonstrated to have protecting effects against exocytotic cell death (Marin et al., 1994). These protective effects have been linked to various mechanisms, including increased expression of neurotrophic factors (Galzi et al., 1996), activated protein kinase C (Li et al., 1999) and inhibition of nitric oxide (NO) production (Shimohama et al., 1996). Nicotine has also been shown to improve cognitive function in a variety of studies in humans and experimental animals (Levin et al., 2006). In particular, α7 nicotinic ACh receptors (α7nAChRs), Ca2+ permeable ligand-gated ion channels expressed primarily in the brain, have been implicated in modulating many cognitive functions like attention and memory (Toyohara and Hashimoto, 2010). α7nAChRs are constituted by five identical transmembrane α7-subunits surrounding a central channel to form an ionotropic receptor. They are located pre-, post-, and extra-synaptically and modulate the release of glutamate (Dickinson et al., 2008; Molas and Dierssen, 2014), GABA (Arias et al., 2010) and dopamine (Quarta et al., 2009). Furthermore, α7nAChRs are directly involved in hippocampal LTP (Mansvelder and McGehee, 2000).

Interestingly, Aβ specifically binds on α7nAChRs when at low picomolar concentrations, whereas, when at high nM levels acts on both α7- and α4β2-nAChRs (Mura et al., 2012). This interaction with α7nAChRs mediates the positive effect of low doses of Aβ on LTP and

memory (Puzzo et al., 2008, 2011). Aβ also affects neurotransmitter release stimulated by the activation of pre-synaptic nAchRs in a dose-dependent fashion (Mura et al., 2012).

Administration of drugs that bind to the α7nAChR (or α7nAChR ligands) has been shown to improve cognitive function in both animal (Levin and Simon, 1998) and human studies (Newhouse et al., 1997, 2004). The main cognitive improvement with these drugs relate to memory, in accordance with the high level of expression of α7nAChRs in the frontal-cortex and hippocampus.

Different α7nAChR agonists and modulators have been investigated for their potential to improve memory and attention disorders encountered for instance in AD and schizophrenia (Toyohara and Hashimoto, 2010). A major drawback of α7nAChRs is that they show rapid desensitization following exposure to agonists (Picciotto et al., 2000). This desensitization is dose dependent, in fact higher doses tend to lead to faster desensitization of these ionotropic receptors.

The identification of high expression levels of neuronal nAChRs in many brain areas, including the hippocampus and cortex, and more specifically of those containing homopentameric α7-subunits, suggested that these receptors might play an important role in cognitive functions (Dani and Bertrand, 2007). This hypothesis was confirmed by the finding that specific agonists of α7nAChRs improved memory performance in animals, as shown initially by the effects of molecules such as GTS-21 (reviewed in: Kem, 2000) and more recently by more selective and potent agonists (e.g. reviewed in: Wallace and Porter, 2011; Posadas et al., 2013). Again, these drugs displayed clear DR curves, i.e. higher doses did not lead to memory improvements or even lead to memory impairments (which may be due to receptor desensitization) stressing the importance of the administrating the right dose in order to get the desired effects.

In humans, different α7nAChR agonists and modulators have been investigated for their potential to improve memory and attention disorders encountered in diseases as AD or schizophrenia (Toyohara and Hashimoto, 2010). Phase I and II clinical trials showed that α7nAChR ligands had beneficial effects on the cognitive function of humans. These improvements related to (episodic) long-term memory as well as working memory and attention (Preskorn et al., 2014; Toyohara and Hashimoto, 2010). Like stated earlier, a major drawback of α7nAChRs is that they show rapid desensitization following exposure to agonists (Picciotto et al., 2000). This desensitization could translate into a quick build-up for tolerance to agonists designed to activate α7nAChRs. Possible causes or functions of desensitization remain elusive. But considering the permeability of α7nAChRs to Ca2+¬, avoiding excitotoxicity might be a function of the desensitization process. Furthermore, since nicotine itself shows low affinity for α7nAChRs, selective α7nAChR agonists are not considered to have addictive properties (Dani and Bertrand, 2007).

Activation of α7nAChRs has also been related with the neuroprotective effect exerted by nicotine (Jonnala and Buccafusco, 2001). The weak partial agonist GTS-21 has been shown to exhibit neuroprotective effects against glutamate-induced and ischemic neurotoxicity (Nanri et al., 1998; Shimohama et al., 1998). Again, these compounds acted in a dose-dependent fashion, with higher doses not leading improvements/increases or even leading to impairments/decreases, again stressing the importance of using the right dose.

It has also been hypothesized that Ca2+ entry through α7nAChRs can promote the survival of neurons (Dajas-Bailador et al., 2000). However, Ca2+ entry is –as mentioned before- also known to be neurotoxic. The degree of Ca2+ influx is probably a very important factor when determining if an outcome will be neuroprotective or neurotoxic. Following this rationale, the neuroprotective effect of partial agonists might be caused by their lower efficacy, suggesting that weaker receptor stimulation may favor neuroprotection (Hogg and Bertrand, 2007), e.g. a more favorable limited Ca2+ influx.

A promising small-molecule selective α7nAChR agonist was called EVP-6124 (encenicline) and was developed for the treatment of cognitive impairment in AD and schizophrenia. EVP-6124 has an excellent brain to plasma ratio and has shown excellent efficacy and potency in a number of preclinical models of cognition (Prickaerts et al., 2012). Also in these studies, clear DR curves with an inverted U-shape have been found. Doses that are too high do not improve cognition, stressing the importance of administrating the right dose for the targeted outcome measure. EVP-6124 appears to be safe and well-tolerated for up to 21 days. In addition, in healthy volunteers, EVP-6124 demonstrated pro-cognitive effects in various cognitive domains including executive function (Preskorn et al., 2014). Last year however, all phase 3 clinical trials of EVP-6124 were terminated due to unwanted side-effects (AD) or insufficient cognitive improvements (schizophrenia). It can be speculated that the failing of these studies might have been caused by insufficient personalized dosing regimens and/or co-application of other receptor ligands (e.g. AChEIs or antipsychotics) that might have interacted with this drug.

3. Glutamatergic system

3.1. Physiological function in learning and memory

Glutamate is the main excitatory neurotransmitter, ubiquitously distributed in the CNS. It is involved in fast neurotransmission as well as in synaptic plastic changes (Watkins and Evans, 1981). Glutamate acts through both metabotropic and ionotropic receptors (reviewed in: Willard and Koochekpour, 2013). The latter, mainly represented by AMPA and NMDA Receptors (AMPARs and NMDARs), are cation channels widely distributed in the cortex and the hippocampus. Native AMPARs are permeable to Na+ and K+ but impermeable to Ca2+ and mediate rapid excitatory transmission. NMDARs are Ca2+-permeable and colocalize with AMPARs in the CNS. NMDARs are sensitive to contemporaneous presynaptic glutamate release and postsynaptic depolarization. At resting potential NMDARs are blocked by the presence of Mg++ in the pore, relieved by postsynaptic depolarization that allows Ca2+ influx in the presence of extracellular glutamate. NMDARs are especially known for their role in LTP. Here, the influx of calcium due to NMDAR activation is thought to activate a number of intracellular signaling pathways that ultimately lead to changes in synaptic efficacy (for a review see Puzzo et al., 2016). Since the impairment of LTP has been widely demonstrated in several models of AD, alterations of the NMDA-mediated glutamatergic transmission have been involved in the pathophysiology of AD (reviewed in Danysz and Parsons, 2012).

Interestingly, the relationship between glutamate and NMDARs is a classical example of physiological molecules behaving in a hormetic fashion. Low doses of glutamate are needed for an acute activation of NMDARs ensuring synaptic transmission and plasticity, whereas high doses are responsible for glutamate excitotoxicity. In fact, a chronic activation of NMDARs leads to neurodegeneration due to the pathological increase of Ca2+ influx into neurons, which in turn triggers neuronal death. Moreover, it has been shown that exposure to low doses of glutamate induced a moderate oxidative stress that might be useful to protect neurons from an eventual exposure to a more severe stress induced by glutamate (Mattson, 2003). Several stresses might induce an increase of glutamate sensitivity, leading to glutamate excitotoxicity. Among this, all the neuropathological hallmarks of AD, such as the increase of Aβ and tau protein, neuroinflammation, oxidative stress and mitochondrial dysfunction have been recognized as potential causes of glutamate excitotoxicity (reviewed in: Danysz and Parsons, 2012).

3.2. Cognitive-enhancing drugs acting on the glutamatergic system

3.2.1. NMDARs antagonists

The above is the rational for the use of NMDARs antagonists, such as memantine, to improve cognition in AD (Bleich et al., 2003;

Shah et al., 2008). Memantine is a low- to moderate-affinity, uncompetitive NMDA receptor antagonist (Tariot et al., 2004) that prevent the excessive binding of glutamate at the NMDARs via their antagonistic mechanism (Tariot et al., 2004; Lanctôt et al., 2009), thus inhibiting the hyperexcitatory activity and preserving and restoring LTP. In October 2003, the FDA approved memantine for the treatment of moderate to severe AD. Memantine is now available in more than 40 countries worldwide, and represents the first member of a new class of medications showing clinical benefit and good tolerability in AD (Tariot et al., 2004). Memantine is the only drug licensed for the treatment of moderate to severe dementia (Namenda) (Lockhart et al., 2009).

Although memantine has been approved for the treatment of moderate to severe AD and dementia, this drug has a rather small effective window. Typical inverted U-shaped DR curves have been reported in the preclinical literature (e.g. Van Dam and De Deyn, 2006). While chronic administration of memantine can have neuroprotective effects, additional research has indicated that memantine also has acute neurotoxic properties at higher doses (Creeley et al., 2008). This neurotoxic effect can be augmented by the simultaneous presence of other drugs (mainly drugs that increase cholinergic activity; see also below) (Creeley et al., 2008). Likewise, cognitive improvement has been associated with doses that are in the right spectrum of the DR curve only in a APP23 mouse model of AD (Van Dam and De Deyn, 2006), whereas higher doses of memantine actually led to cognitive impairment. This again shows that the right dosing regime is imperative when acting on these systems.

As additional information, also glutamate metabotropic receptors (mGluRs) have been involved in AD pathogenesis. In particular, preclinical and clinical studies have demonstrated a downregulation of mGluR1 and an upregulation of mGluR2 in AD (Bruno et al., 2001; Albasanz et al., 2005). However, mGluRs ligands have not shown efficacy, at now, in AD models.

4. Nitrinergic transmission

4.1. Physiological function in learning and memory

Nitric oxide (NO) is a gaseous molecule able to freely cross the cellular membrane by diffusion. It is synthesized by the conversion of the aminoacid L-arginine in L-citrulline by the enzyme NO synthase (NOS). There exist different isoforms of NOS, classified as the constitutive forms (cNOS) and inducible form (iNOS). cNOS comprise the neuronal form (nNOS) or type I, widely expressed in the brain, especially in the cortex and hippocampus, and the endothelial form (eNOS) or type III, expressed in the endothelium. cNOS produce low amounts of NO (nM range) for a short period (seconds-minutes) in physiological conditions and require a binding with calmodulin, accomplished only when local calcium levels are elevated. iNOS, or type II, is present in smooth cells, macrophages, hepatocytes, glia and endothelium, and produces high amounts of NO (μM range) for a long period (hours-days) in response to immunological stimuli; it is calcium-insensitive, so that it can lead to the production of NO even at low calcium levels (reviewed in: Puzzo et al., 2006).

Once formed, NO diffuses by concentration gradient and combines with its biological receptor, soluble guanylyl cyclase (sGC), that, in turn, provokes an increase in cGMP levels triggering a series of chain reactions able to induce a biological response.

NO might also act in a cGMP-independent way, by mechanisms of protein nitrosation and nitration. Moreover it can exert an antioxidant resulting in a neuroprotective function against oxidative stress (Chiueh, 1999).

The ability of NO to induce biphasic DR has been widely studied in both physiological and pathological conditions (reviewed in: Calabrese et al., 2007). Indeed, NO is considered the par excellence “Janus” molecule, which might be neurotoxic or neuroprotective depending upon the amount produced and the NOS involved, (i.e. cNOS versus iNOS).

In the CNS, NO has been widely studied for its ability to act as a retrograde messenger mediating a peculiar form of bidirectional neuronal communication in which information are transferred from the post-synaptic to the pre-synaptic neuron. In particular, NO has been involved in LTP where the NMDA-mediated increase of Ca2+ flow stimulates nNOS to release NO that in turn, spreads across neurons. Interestingly, NO seems to regulate NMDARs activity (Lipton et al., 1993; Choi et al., 2000) and it might be neuroprotective against NMDA-induced excitotoxicity (Lipton et al., 1993).

The role of NO in learning and memory has been investigated by using NOS inhibitors, such as an inactive analogue of the L-arginine (L-NAME), thereby preventing NO release, or by treatment with exogenous substances that actively react with NO, blocking it, before any reaction with biological target molecules. Behavioral studies have demonstrated that NO is needed for different types of memory (Chapman et al., 1992; Böhme et al., 1993; Prickaerts et al., 1997; Zou et al., 1998; Kirchner et al., 2004; Koylu et al., 2005). Moreover, low doses of NO are able to convert the protein-synthesis independent early phase of LTP (E-LTP) into the protein-synthesis dependent late-phase (L-LTP), i.e. short-term into long-term memory (O'Dell et al., 1991; Lu et al., 1999). However, other studies have demonstrated that exogenous application of NO is able to induce or decrease plasticity depending upon experimental conditions and variables such as concentration and time of application (reviewed in: Puzzo et al., 2006).

Several studies have indicated that NO action in synaptic plasticity and memory is mediated by the cGMP/PKG pathway since drugs able to increase cGMP levels or to stimulate or PKG enhances LTP and memory performance, whereas inhibition of the pathway blocks synaptic plasticity and memory (reviewed in Thatcher et al., 2005). Activation of PKG, in turn, leads to phosphorylation of transcription regulating factors such as cAMP response element binding protein (CREB) at Ser133, a critical event in both LTP and the establishment of long-term memory (Lu et al., 1999; Lu and Hawkins, 2002). NO also mediates CREB–DNA binding via a Ser133- independent mechanism by the S-nitrosylation of nuclear proteins associated with CREB target genes (Riccio et al., 2006).

On the other hand, an excess of NO becomes harmful (Pacher et al., 2007) leading to the production of toxic compounds known as “reactive nitrogen species”, such as peroxynitrite, highly reactive and responsible for the NO-mediated cell death and neurodegeneration. Indeed, an excessive production of NO by iNOS has been involved in the typical neuroinflammation occurring in AD and Parkinson’s disease (reviewed in: Calabrese et al., 2007). Intriguingly, various studies have demonstrated that Aβ-induced neurotoxicity is supported by an increase of iNOS activity and NO production (Tran et al., 2001; Haas et al., 2002; Xie et al., 2002). On the contrary, other studies have underlined a protective role of NO, so that a stimulation of the NO/cGMP pathway protected against the Aβ-induced cell death and impairment of synaptic plasticity and memory (Baltrons et al., 2002, 2004; Puzzo et al., 2005, 2009a, 2009b, 2014; Zhang et al., 2013). These findings supported the idea that NO behaves in a hormetic fashion, exerting two opposite effects, neuroprotective and neurotoxic, depending upon the dose. In particular, an excessive production of NO catalyzed by the iNOS present in the microglia is involved in the neuronal damage characteristics of the typical neuroinflammation present in AD; whereas, low quantity of NO, as catalyzed by nNOS and eNOS, are able to exert a neuroprotective effect. This is also consistent with the decrease of cNOS and the increase of iNOS found in aged rats (Law et al., 2002), or in stressed animals (Palumbo et al., 2007) and with the increase of Aβ and cognitive dysfunction in eNOS KO mice (Austin et al., 2013). Moreover, the NO/cGMP/PKG/CREB system is impaired after an exposure to Aβ, in animal models of AD or in the cortex of AD patients (Bonkale et al., 1995; Baltrons et al., 2002; Venturini et al., 2002; Puzzo et al., 2005, 2009; Miller et al., 2010), and, on the other

hand, a stimulation of the NO/cGMP/PKG pathway rescued the Aβ-induced impairment of LTP, memory and CREB phosphorylation (reviewed in: Teich et al., 2015).

Thus, production of excessive amounts of NO might induce neuropathology, whereas small increases are needed for fundamental physiological processes and might have a therapeutic effect.

Interestingly, the NO/cGMP cascade is strictly related to Ach and glutamate systems described in the previous paragraphs. For example: i) activation of mAchRs in the hippocampus induced an increase of cGMP (Sirviö, 1999), whereas inhibition of cGMP blocked mAchRs activation (Krause and Pedarzani, 2000); and ii) inhibition of NMDARs can be rescued by L-Arg, NO donors or cGMP analogs (Yamada et al., 1996).

4.2. Cognitive-enhancing drugs acting on the NO/cGMP system

4.2.1. NO mimetics

Based on these findings, several drugs acting on the NO/cGMP pathway have been proposed for the treatment of cognitive impairment in AD and other disorders characterized by memory impairment. Nitrates such as nitroglycerin, classically used for treatment of angina pectoris, are contraindicated in AD for their peripheral effects on the cardiovascular system. Hybrid nitrates such as NO-NSAIDs (NO donor + a non-steroidal anti-inflammatory drug) or novel nitrates (GT715 and GT1061) have been proposed to increase cGMP in the brain and in models of brain injury and neurodegeneration, with minimal effects on cardiovascular system (reviewed in: Thatcher et al., 2005).

4.2.2. Phosphodiesterase inhibitors

Another strategy aimed to stimulate the NO/cGMP pathway is to block the degradation of cGMP by using phosphodiesterase inhibitors (PDE-Is). Phosphodiesterases (PDEs) play an important role in signal transduction since they are the only enzymes that degrade the cyclic nucleotides cAMP and cGMP in response to intracellular stimuli. Therefore, PDE-Is diminish the degradation of cAMP and/or cGMP (Beavo, 1995), inducing the elevation of one or both second messenger molecules. So far, over 100 human PDEs have been described, divided into 11 families that differ in brain distribution and substrate specificity (Bender and Beavo, 2006). Specifically, PDE1, PDE2, PDE3, PDE10, PDE11 have a dual substrate specificity, hydrolyzing both cAMP and cGMP. PDE4, PDE7 and PDE8 are cAMP specific; PDE5, PDE6 and PDE9 are cGMP specific. In the past decades, a growing body of studies showed the cognitive enhancing effects of PDE-Is in healthy, aged and transgenic mice models of AD (reviewed in Heckman et al., 2015). Here, we will pinpoint the importance of DR curves before determining the appropriate dose for a pharmacologic intervention with a PDE-I.

PDE1 is a Ca^{2+}-activated PDE with dual substrate specificity hydrolyzing both cAMP and cGMP. In the past two decades there has been an increased interest for the development of specific PDE1-I for cognitive improvement. Recently, it has been shown that the specific PDE1-I ITI-214 improved memory acquisition, consolidation and retrieval in object recognition task (ORT) in healthy rats after acute oral treatment (Snyder et al., 2016). Specifically, a DR curve ranging from 0.1 to 10 mg/kg, showed a maximum response at 1 mg/kg in acquisition, retrieval and late consolidation, whereas early consolidation was improved with 3 mg/kg. These results stress out the importance of testing multiple doses in order to determine the appropriate efficacious dose to enhance a specific cognitive process in a specific dose window.

As PDE1, PDE2 has a dual substrate specificity hydrolyzing both cyclic nucleotides. BAY60-7550 was the first selective PDE2-I developed that improves memory consolidation in normal healthy mice and rats tested with the ORT and the social recognition task. Importantly, the positive effect of BAY60-7550 had a DR curve that differed between mice and rats, i.e. rats needed a higher dose probably because of discrepancy in different pharmacological and pharmacokinetic properties of BAY60-7550 between species (Boess et al., 2004). Additionally, chronic administration of the lowest effective dose of BAY60-7550 enhanced cognitive performance in the APPswe/PS1dE9 mouse model for AD disease without inducing anxiety or depressive-like behavior (Sierksma et al., 2013).

PDE4 hydrolyzes specifically cAMP and its inhibition has been extensively investigated as a potential strategy for cognitive enhancement. Among the PDE4-Is, rolipram has a prominent position and several studies showed its cognition enhancing effects in young, old and AD transgenic animals. Interestingly, one of the first studies demonstrated that lower doses of rolipram (0.03 mg/kg) improved retention in healthy, young and aged mice in a contextual fear conditioning task, whereas higher doses (0.8 mg/kg) were ineffective (Barad et al., 1998). Several studies confirmed that low doses of PDE-Is, including rolipram and rofumilast, enhanced memory consolidation in rats (Rutten et al., 2006; Bruno et al., 2011; Vanmierlo et al., 2016). A chronic administration of the same concentration was also able to rescue synaptic plasticity and hippocampal-dependent memory in the APP/PS1 mouse model of AD (Gong et al., 2004; Costa et al., 2007), and to reduce tau levels in a mouse model of tauopathy (Myeku et al., 2016).

The newly synthetized and potent PDE4-I, GEBR-7b, enhanced memory in both mice and rats at a dose of 0.003 mg/kg in the ORT (Bruno et al., 2011), and at a lower concentration of a 0.001 mg/kg it was able to attenuate the memory deficit in APPswe/PS1dE9 mice after chronic treatment (Sierksma et al., 2014).

The effect of rolipram has also been tested in rhesus monkeys. Ramos and colleagues showed that rolipram improved spatial memory in young monkeys, whereas it did not have any effect on aged monkeys (Ramos et al., 2003). Noticeably higher doses impaired their working memory, suggesting that higher doses may have deleterious instead of positive effects (Ramos et al., 2003; Arnsten et al., 2005). This might be due to the overstimulation of the already disinhibited cAMP/PKA signaling pathway in the prefrontal cortex (Ramos et al., 2003; Arnsten et al., 2005). In this scenario, PDE inhibition might have negative effects on cognition and plasticity when PDEs are already down-regulated, as happens in the aged brain where cAMP levels and PKA activity are high due to a possible compensatory mechanism. Therefore, with aging the DR curve appears to shift to the left and lower doses should be examined.

Other studies performed on cynomolgus macaques showed that rolipram improved executive functioning at different doses for male and female monkeys, emphasizing possible gender differences (Rutten et al., 2008; Sutcliffe et al., 2014).

Although there are promising studies regarding the cognitive enhancing effects of rolipram in rodents, adverse side effects including emesis in humans greatly hampered clinical development of PDE-Is. The unwanted effects were due to the fact that rolipram is a PDE4-I that non-selectively inhibits all four isoenzymes of PDE4 (A-D), of which B and D are implicated in emetic effects (Robichaud et al., 2002a). However, because PDE4D is the major isoform implicated in cognition (Gurney et al., 2015), more specific or selective PDE4-Is have been designed in an attempt to diminish the side effects. One approach involved the development of allosteric modulators of PDE4D that partially inhibit the enzymatic activity of PDE4. Since rodents cannot vomit, the reduction of anesthesia time in the xylazine/ketamine test has been proposed as a behavioral equivalent for the assessment of emetic-like effects of PDE4-Is (Robichaud et al., 2002b). Indeed the allosteric modulator D159687 decreased emetic potency without affecting the efficacy in cellular and in vivo models (Burgin et al., 2010). Specifically, D159687 showed to elicit emesis at a 60 times higher dose than the lowest effective one able to enhance cognition (Burgin et al., 2010; Sutcliffe et al., 2014).

A recent study indicated that roflumilast, which is considered to non-selectively inhibit all PDE4 isoforms, has the potential to improve cognition without emetic side effects, since the active dose used in the

memory tests was 30–100 times lower than the emetic-inducing dose in mice (Vanmierlo et al., 2016). Moreover, the second generation novel PDE4-I GEBR-7b, a full inhibitor for PDE4D, showed to improve memory performance in healthy mice and rats with an active dose at least 100 times lower than the one affecting the surrogate measurements for emesis in rodents (Bruno et al., 2011).

To summarize, the future of PDE4 as a drug target for cognitive enhancement and neuroprotection lies in development of allosteric modulators and highly selective inhibitors of PDE4, which have the same denominator to increase the distance between the therapeutic and the emetic window of the drugs and thus pulling apart the cognitive and the emetic effects. Once again, this underlines the importance of constructing full DR curves.

PDE5 exhibits high specificity for cGMP and the initial development of selective PDE5-Is, such as sildenafil, vardenafil and tadalafil, was aimed to treat erectile disorders (Setter et al., 2005). Nevertheless, there is a plethora of studies showing the cognitive enhancing effect of PDE5-Is. In rats, sildenafil improved memory consolidation in the ORT with a peak at 3 mg/kg (Prickaerts et al., 2002) and memory acquisition with a highest effective dose at 10 mg/kg (Prickaerts et al., 2005). In mice, the peak dose able to induce cognitive enhancing effects on memory consolidation was 1 mg/kg (Rutten et al., 2005), again underlying possible differences in pharmacological and pharmacokinetic properties of sildenafil between different species. Finally, sildenafil improved recognition memory in the male cynomolgus macaque at the increasing doses of 1 and 3 mg/kg (Rutten et al., 2008).

Some studies have shown positive effects of sildenafil after chronic administration in transgenic mouse models of AD and aged mice. Puzzo et al. (2009, 2014) were the first to show that chronic administration of sildenafil rescued synaptic plasticity and memory in APP/PS1 mice. DR curves showed that the minimum effective concentration was 3 mg/kg daily for 2 weeks. This improvement was paralleled by an increase in CREB phosphorylation and a reduction in Aβ levels. The same dose was able to reverse pathological features related with aging, inducing a reduction of pro-apoptotic proteins and Aβ levels, with an increase of plasticity-related molecules (Puzzo et al., 2014).

The growing field of studies regarding the cognitive enhancing action of PDE4-Is and PDE5-Is raises a discussion regarding the mechanism of action of the above inhibitors. The increase of cAMP and cGMP might induce a cognitive enhancing effect via the increase of cerebral blood flow and glucose delivery to the brain (Paternò et al., 1996). However, the dose needed to improve cognition is below the dose required to induce vascular and metabolic effects. Additionally, previous data showed that the cognitive enhancing properties of rolipram and vardenafil in object recognition and spatial memory were not related to any main effects on blood flow and glucose utilization in the rat brain (Rutten et al., 2009). Finally, there are studies showing that the positive effects of sildenafil (Puzzo et al., 2009, 2014; Zhang et al., 2013) and rolipram (MacKenzie and Houslay, 2000; Monti et al., 2006) on cognition are related to activation of pathways that lead to increases in CREB phosphorylation. Altogether the above observations indicate that cognitive enhancing effects of PDE4-Is and PDE5-Is are attributed to synaptic plasticity related mechanisms rather than cerebrovascular effects. Once again, this demonstrates that separate DR curves are essential to clearly distinguish between the different mechanisms of actions and functional effects of a drug.

Like PDE4, PDE7 is a cAMP-specific hydrolyzing enzyme. A recent study have shown that chronic administration of the PDE7-I, S14 ameliorated memory impairments, and reduced Aβ deposition and tau phosphorylation in APP/PS1 mice (Perez-Gonzalez et al., 2013).

PDE9 is highly selective for cGMP. So far, two studies have been conducted with the selective PDE9-Is BAY73-6691 and PF-04447943 showing their positive effect on cognition in healthy rodents at doses ranging from 0.03 to 3 mg/kg, depending upon the species (rats vs. mice) and the task (ORT, Y-maze) (van der Staay et al., 2008; Hutson et al., 2011).

PDE10 has a dual specificity hydrolyzing both cAMP and cGMP. Because of the high expression of PDE10A mRNA in the striatum (Seeger et al., 2003), PDE10-Is were studied as antipsychotics and potential cognitive enhancers in animal models of schizophrenia. Papaverine, TP-10 and MP-10 are the most well-known PDE10-Is. However, results are conflicting because papaverine has been found to impair or improve cognition depending upon the dose and the task used to evaluate memory performances (Hebb et al., 2008; Grauer et al., 2009). There is also some controversy regarding the efficacy of antipsychotic effects of PDE10 inhibition, since blockage of striatal dopaminergic signaling could evoke extrapyramidal symptoms (Seeman and Tallerico, 1999).

5. Combination treatments

Combinations of different drugs may ultimately prove to be the most productive strategy to treat cognitively impaired patients (Giacobini, 2003). Examples of such combinations will briefly be described in this section. Most studies deal with combining a promising drug with the AChEI donepezil as the latter is generally considered as the gold standard in the clinic (e.g. the α7nAChR agonist EVP-6124 and donepezil; Prickaerts et al., 2012), or the PDE4 inhibitor roflumilast with donepezil (Vanmierlo et al., 2016). Another example is the combination including the AChEI/nAChR ligand galantamine with the NMDA antagonist memantine which improved cognitive outcome measures in rats (Nikiforuk et al., 2016). A promising approach is also to combine drugs of the same class, e.g. a PDE4 inhibitor together with a PDE5 inhibitor (Bollen et al., 2015). The purpose of combining drugs is not merely to be additive, but actually to be synergistic. Thus, the cognitive enhancing effect of the combination therapy is greater than simply adding up the effects of the separate treatment. For this purpose in the above mentioned preclinical combination studies, sub-efficacious doses of the respective treatments are associated to evoke a synergistic effect. In this way, dose limitations of the separate treatments, which are mostly due to primarily autonomic gastrointestinal side-effects including nausea and emesis, are circumvented with an increased efficacy for neuroprotection and cognitive enhancement.

Understanding the mechanism of action for increased efficacy when combing drug is obviously essential. The interaction between α7nAChR agonist EVP-6124 and ACh, which is increased after donepezil treatment can serve to illustrate this phenomenon. The interaction at the cellular level was investigated in sustained exposure experiments that were aimed at mimicking the conditions of an animal treated with EVP-6124. The results clearly illustrated that sustained exposure to a concentration of EVP-6124 below 1 nM potentiated the ACh-evoked current. Increasing the EVP-6124 concentration to 3 nM or above, caused a marked reduction of the ACh-evoked current that was attributable to receptor desensitization. Of note, a concentration lower than 3 nM of this drug alone was insufficient to activate the receptor in in vitro studies (Prickaerts et al., 2012).

Although different mechanisms can be postulated to account for the observed potentiation, the simplest and most probable model considers the co-agonistic behavior of EVP-6124 and ACh at α7nAChRs. In this model, a single receptor displays at least two pockets where the ligand can bind (Cachelin and Rust, 1994). Similarly, two molecules of ACh must bind to the α7nAChR to activate it. Exposure to a low concentration of a high affinity ligand, such as EVP 6124, will increase the probability that a single molecule of this ligand occupies the receptor. As occupancy of the receptor by a single molecule is assumed to be insufficient to activate the receptor, exposure to such a low concentration of ligand is not expected to cause channel opening. Brief and intermittent exposure to another ligand with lower affinity, such as ACh, will then trigger channel opening and an inward current (Prickaerts et al., 2012).

The finding of a novel mechanism of action of a partial agonist acting at a concentration in the sub-nanomolar range through a co-

agonistic mechanism may lead to a more desirable side-effect profile in respect of classical approaches which dictate that the drug should be dosed to full agonist concentrations. Activation of α7nAChRs by exposure to a low agonist concentration of a drug such as EVP-6124 exploiting a co-agonist mechanism, is expected to increase the drug safety margin, to minimize undesired interactions with other receptors, and to open new and promising therapeutic avenues in combination with classical AChEIs at lower than typically prescribed doses. Lower doses of AChEIs will most likely also result in less, or better tolerable side-effects. Furthermore, desensitization of α7nAChRs, and hence tolerance to these drugs, might not occur at concentrations in the sub-nanomolar range, again stressing the importance of dosing in the appropriate spectrum of inverted-U dose response curves (Prickaerts et al., 2012).

Also, different human studies have investigated the effects of drug associations, for instance memantine with an AChEI for the treatment of AD. It has been found that in patients with moderate to severe AD, who were receiving stable doses of donepezil, memantine resulted in significantly better outcomes than placebo (or donepezil alone) on measurements of cognition, activities of daily living, global outcome, and behavior. There were, for example, fewer behavioral disturbances and psychiatric symptoms in patients in the memantine group. Furthermore, this combination was safe and well tolerated by the subjects with an administered memantine dosage of 20 mg per day (Tariot et al., 2004). This research also suggests that there are additive or even perhaps synergistic effects of memantine in combination with donepezil. Importantly, it is to be assumed that there are no pharmacokinetic or pharmacodynamic interactions observed between the combined drugs, which was the case for memantine and donepezil in healthy volunteers (Periclou et al., 2003).

Interestingly, a preclinical study investigating the combined effects of memantine and donepezil, found that the latter markedly potentiated neurotoxicity induced by memantine in the rat brain. In this study, it was found that a relatively high (still considered neuroprotective) dose of memantine caused an acute mild neurotoxic reaction in the adult rat brain. When combined with a relatively high (still considered cognitive enhancing) donepezil, this neurotoxic reaction was potentiated (Creeley et al., 2008). This indicates (again) that when combining drugs, it is of great importance to use the appropriate (low) doses.

6. Potential pitfalls when performing a DR curve

DR curves appear to be necessary to better understand the profile of physiological molecules as well as drugs. However, data are often conflicting because many factors might influence the experimental setting (Davis and Svendsgaard, 1990).

6.1. Pharmacokinetics: timing and duration of treatments

When studying memory, the time point of administration has a particular relevance especially when administered acutely to influence a specific cognitive process. For instance, depending on the pharmacokinetics of a drug (see also below) one might influence memory consolidation processes, while the drug was given before learning with the intent to specifically influence acquisition (van Goethem et al., 2012, Akkerman et al., 2015).

A DR curve after chronic treatment is obviously different from the one obtained after acute treatment as they tap into different mechanisms of action, i.e. cognitive enhancement versus a neuroprotective mechanism. The former involves neurotransmitter release and improved signal transduction pathways. The latter involves more structural changes including the formation of new neurons. Of note, formation of dendrites and synapses can be influenced by both treatments. Interestingly, most chronic studies do not refer to the half-life of a drug and only use a once or twice daily administration. When pharmacokinetic measurements are lacking it is difficult to ascertain whether steady-state plasma levels have been achieved. In general, this is easiest done by injecting approximately 5–6 times at the half-life of the drug (Advokat et al., 2014).

Inverted U-shaped curves are also different between studies when using a different route of administration, e.g. p.o. vs. i.p./s.c.. This will have an effect on the pharmacokinetics of a drug and thus the points mentioned above become applicable.

6.2. Housing

An often-neglected aspect causing differences in DR curves is the animals' housing condition, including factors as social housing, cage enrichment or physical activity. Rodents and monkeys are social animals and standard (solidary) housing is considered stressful and therefore not allowed anymore, unless the researcher has an explicit reason to do so. To further increase animal welfare, cage enrichment (e.g. toys, nesting material) has become also standard (Wolfer et al., 2004), unless the researcher can show it interferes with the study.

Animals living in an enriched environment (social, enriched housing and/or increased physical activity) have more 'plastic' brains, as demonstrated by an increase of CREB activation, the higher number of dendrites and neurons, and the improvement of cognitive performance (Simpson and Kelly, 2011). Conversely, standard housed animals could present a brain impoverishment. Thus, it seems plausible that drugs can have differential effects in animals with different housing conditions.

To our knowledge, cognitive enhancers have not been investigated thoroughly in this respect, yet studies with antidepressants and psychostimulants already demonstrated differential functional effects (e.g. Yildirim et al., 2012). Linked to this it has actually been argued that many drug targets might have been missed by using standard housing and inactive laboratory animals (Gurwitz, 2001).

To explore the possible influence of an enriched environment we recently tested the cognitive enhancing effect of an acute injection with the PDE5-I vardenafil in standard (solidary), socially or environmentally-enriched (social housing, toys and physical activity) housed animals (Akkerman et al., 2014). Our data suggested that the DR curve shifts to the left, i.e. the optimum dose was lower, depending upon the housing conditions. This was likely due to the enhancement of CREB activation after increased enrichment (socially as well as environmentally). Interestingly, vardenafil effectiveness was reduced in the environmentally-enriched housed animals, probably because it was not able to further increase CREB signaling in these rats. This observation should be taken into consideration when studying the effect of a cognitive enhancer in human healthy adults, who already perform optimally. Based on the above, we argue that animals with an enriched environment (social housing, cage enrichment and/or physical activity) could be considered as a more valid and predictive model for the evaluation of cognitive enhancing drugs than standard housed animals. Moreover, one has to be aware that the DR curve would probably shift to the left.

6.3. Translation

Numerous formulas exist to calculate the effective dose of a drug for humans based on animal dosing (e.g. Reagan-Shaw et al., 2008). Yet many times such dose does not appear to be predictive as differences in pharmacokinetic (bioavailability) properties arise and complicate dose translation. An example is for instance the α7nAChR agonist EVP-6124 (encenicline) having a very short half-life in rodents and a very long half-life in humans (Prickaerts et al., 2012; Barbier et al., 2015; Deardorff et al., 2015; van Goethem et al., 2015). Obviously, one even should check for possible differences in pharmacokinetic properties between gender, age or even when being fed (type of food) or fasted.

Linked to this, the DR curves obviously are not these same when

translating doses from animals to humans. Another complicating issue is when the effective DR range is very narrow in animal studies. An example is the PDE-I which shows mostly only one to two optimum doses when using a log scale (e.g. Rutten et al., 2009; Vanmierlo et al., 2016). This makes it difficult to titrate the effective dose for human studies. Therefore it is advised to test at least three doses in a human study to optimize the chance of success for finding the optimum dose. Although it is costly, it helps to avoid entering discussing explaining the lack of efficacy of a drug. An example of this could be the study in which the PDE9 inhibitor PF-04447943 was tested to evaluate its effects on cognitive symptoms in patients with moderate to mild AD. 25 mg dosing twice daily during twelve weeks had no effects on cognition in these patients (Schwam et al., 2014). It was suggested that the treatment duration may not have been long enough and/or a less detrimental population as for instance age-associated cognitive impaired subjects could be a better choice for treatment (Kleiman et al., 2012). However, it could also be speculated that the single dose tested was not the appropriate one (too low or too high).

Another point, which has to be taken into consideration, is that possible lacks in face validity of many animals models and tests of cognition (e.g. Blokland et al., 2014; Blokland et al., 2015). For instance, animal tests are non-verbal, whereas human test are in particular verbal. This greatly decreases the chance of finding an optimum dose of a drug or even a drug effect at all in humans.

7. Conclusions

In conclusion, to optimize the chance of finding an effect of a drug on cognitive enhancement and neuroprotection, it is suggested to test at least three doses using a Log scale (covering a 10 fold dose range). Obviously more doses are preferable with a maximum up to 5 when needed to cover a 100 fold dose range.

Besides dosing, it is obvious that more factors have to be taken into consideration of which the two most essential ones are timing (when) and duration (how long) of treatment. Nevertheless, we argue that when finding initially no effect of a drug treatment, the first factor to control for, is checking whether the appropriate dose or doses have been chosen. And though many times the first reflex appears to be to choose for increasing the dose, we argue to strongly consider the option of actually lowering dosing.

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