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A computerized test battery to study pharmacodynamic effects on the central nervous system of cholinergic drugs in early phase drug development.

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Abstract:	Investigating potential pharmacodynamic effects in an early phase of central nervous system (CNS) drug research can provide valuable information for further development of new compounds. A computerized and thoroughly validated battery of neuropsychological and neurophysiological tests has been shown to be sensitive to detect drug-induced effects of multiple new and existing compounds. The test battery covers the main CNS domains which have been shown to respond to drug effects and can be repeatedly administered following drug administration to characterize the concentration-effect profile of a drug. The standard tests in the battery are saccadic eye movement, smooth pursuit eye movement, the Bowdle visual analog scale (VAS), the Bond and Lader VAS, body sway, adaptive tracking, visual verbal learning, and quantitative electroencephalography. However, the test battery is adaptive in nature, meaning that it can be composed and adjusted with tests fit to investigate specific drug classes, or even specific receptors.

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Author Comments:	Dear Editor, As you can see in the manuscript we have chosen to describe the quantitative EEG procedure as it i was part of the protocol and is part of the core test-battery of the NeuroCart computer system. However, as the EEG procedure is standard and similar to how EEG is measured in other research settings we do not feel that it has an added value to include it in the script (i.e. yellow highlighted text). Best wishes, Ellen
Additional Information:	
Question	**Response**
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CHDR

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United States of America

Att. Dr. Alisha DSouza

Date: 30-Aug-2017
Ref.: Cover Letter manuscript re-submission

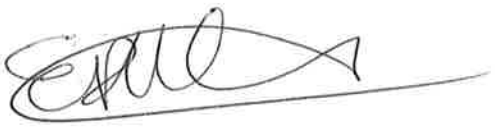
Dear Dr. DSouza, dear Alisha,

With this letter I would like to re-submit the manuscript entitled '*The NeuroCart: studying pharmacodynamic effects of cholinergic drugs in early phase drug development*' to the Journal of Visualized Experiments on behalf of the other authors.
We would like to thank the editor and the reviewers for their review of our submission. We have rewritten and updated the manuscript and the tables and figures following the suggestions and comments made and hope that the submission meets your criteria for publication.

In this re-submission you will find the new manuscript as well as a tracked changes version. In the response to reviewers document you will find our detailed answers to the questions and comments.

We are looking forward to your response regarding our re-submission, and are happy to answer any questions that might rise.

Kind regards,



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TITLE:

A Computerized Test Battery to Study Pharmacodynamic Effects on the Central Nervous System of Cholinergic Drugs in Early Phase Drug Development

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KEYWORDS:

Drug development, central nervous system, pharmacodynamics, neuropsychology, neurophysiology, neuropsychopharmacology, computerized test battery, challenge model.

SHORT ABSTRACT:

A validated computerized battery of neuropsychological and neurophysiological tests is used to study pharmacodynamic effects on the central nervous system of newly developed drugs in early phase development. To demonstrate the test battery, the acute effects of mecamylamine and the reversal of these effects by two agonist drugs are described.

LONG ABSTRACT:

Investigating potential pharmacodynamic effects in an early phase of central nervous system (CNS) drug research can provide valuable information for further development of new compounds. A computerized and thoroughly validated battery of neuropsychological and neurophysiological tests has been shown to be sensitive to detect drug-induced effects of multiple new and existing compounds. The test battery covers the main CNS domains, which have been shown to respond to drug effects and can be repeatedly administered following drug administration to characterize the concentration-effect profile of a drug.

The standard tests in the battery are saccadic eye movement, smooth pursuit eye movement, the Bowdle visual analog scale (VAS), the Bond and Lader VAS, body sway, adaptive tracking, visual verbal learning, and quantitative electroencephalography (qEEG). However, the test

battery is adaptive in nature, meaning that it can be composed and adjusted with tests fit to investigate specific drug classes, or even specific receptors.

Showing effects of new cholinergic drugs designed to have a pro-cognitive outcome has been difficult. The pharmacological challenge model is a tool for early proof-of-pharmacology. Here, a marketed drug is used to induce temporary and reversible disease-like symptoms in healthy subjects, via a pharmacological mechanism related to the disease that is targeted as indication for the new compound. The test battery was implemented to investigate the potential of the nicotinic receptor antagonist mecamylamine to be used as a challenge model for cholinergic dysfunction, as seen in neurodegenerative disorders.

A worsening of scores in a dose dependent manner on the visual verbal learning test (VVLT; a test for learning and memory abilities) and the adaptive tracking test (a measure of visuomotor control and arousal), in particular, showed that the test battery is sensitive to showing acute pharmacodynamic effect after administration of anti-cholinergic drugs.

INTRODUCTION:

With human life expectancy steadily increasing over the last century the prevalence and incidence of diseases of the aging brain, such as dementia and other neurodegenerative processes, also grow. In parallel, the development of new drugs to treat these diseases is therefore expanding. However, many new drugs intended to be active in the CNS fail to reach the market due to lack of central effects or unwanted side effects in later phases of drug development[1]. In traditional phase 1 studies the objectives are to gain information on the pharmacokinetics, that is, the effect that the human body has on the drug (for example by metabolizing), as well as safety and tolerability of the new drug. Early proof of pharmacodynamic effect (the effect that the drug has on the body), however, may be even more important in decisions on moving forward in the clinical development of a new compound and may help avoid erroneous decision making with consequences at later phases of the development process[2].

In the past two decades, the Centre for Human Drug Research (CHDR) has developed a computerized test battery of neuropsychological and neurophysiological measurements sensitive to CNS effects of drugs. This test battery is used repeatedly over the day to measure pharmacodynamic effects of a new compound. It thereby provides evidence of the drug's ability to have the desired effect, to penetrate the blood-brain barrier and enter the brain, or the lack thereof[3]. Also, outcomes of the test battery could provide information on the mechanism of action of a compound as the individual tests correspond to specific drug-responsive CNS domains. For example, if effects of the new drug are seen on the maze learning test, which is a test for visuospatial working memory, this could indicate that the drug acts on receptors in parts of the brain involved in visuospatial working memory. In addition, the test battery is used to screen for CNS side effects for compounds that are not designed to work in the CNS, and where CNS activation needs to be ruled out.

The test battery is made up of a large number of cognitive and neurophysiological tests, which have been shown to be sensitive to detect pharmacodynamic effects of CNS active drugs[3-6]. The

core test battery comprises six neuropsychological domains: executive functioning, attention, memory, visuomotor functioning or coordination, motor skills, and subjective drug effects. The core tests are: saccadic eye movement[7], smooth pursuit eye movement[8], the Bowdle VAS[9], the Bond and Lader VAS[10], body sway, adaptive tracking[11], visual verbal learning[12], and qEEG, which cover the main cognitive and neurophysiological domains mentioned earlier. These tests have been shown to be able to measure changes in CNS functions as a result of administration of several types and classes of drugs (see below). The battery can be repeatedly administered (up to 12 times following drug administration) due to the 30-min total administration time, which is essential to characterize the concentration-effect profile of a drug. The test battery can be expanded and adjusted with different tests fit to investigate specific drug classes, or even specific receptors. The test battery has been validated in a wide range of drugs acting on different CNS systems (*e.g.,* benzodiazepines, antipsychotics, ethanol, and cannabis[12-21]) to be able to reliably demonstrate drug related CNS effects.

While other computerized test batteries exist (described for example in Egerhazi *et al.*[22] and Underwood *et al.*[23]), and are widely used in clinical trials, the test battery described in this paper stands out as it not only includes neuropsychological tests such as the VVLT and the VASs, but also neurophysiological measurements (*e.g.,* EEG, eye movement tests), thereby combining different aspects of brain functioning in one test battery, and better reflecting the multimodal nature of cognitive behavior. Furthermore, as the test battery is computerized, the test results are generated electronically. This results in outcome values that are the same when used in different studies by different research staff, allowing for standardization of results, as well as values that are less error prone compared to scoring by hand. The outcome files can be easily uploaded into electronic database systems and can be used to generate interim reports of the pharmacodynamic effects of new drugs within a day.

There is at least one class of drugs where early proof of pharmacological effect in the brain has been difficult; the (pro)cholinergic drugs. Acetylcholine is one of the main neurotransmitters of the CNS and has been shown to play a key role in cognition, specifically in processes such as learning and memory[24,25]. Consequently, cholinergic dysfunction is indicated to underlie neurodegenerative processes such as Alzheimer's disease[26]. New compounds designed to enhance cognitive functioning, such as muscarinic and nicotinic receptor specific agonists, are now entering clinical studies.

As early phase studies are usually performed in healthy, often young subjects who cognitively perform at a normal level, it is difficult to study or even show proof of pharmacodynamic effect of a new drug intended to treat cognitive decline in patients with a disease of the brain.

Our group has therefore developed a tool that can be used for demonstrating early proof of pharmacology of a new drug: the pharmacological challenge model. An already approved and marketed drug is used to induce temporary and reversible disease-like symptoms in healthy subjects, via a pharmacological mechanism related to the disease that is targeted as indication for the new compound. In most cases this effect is an unwanted side effect of the drug, resulting from activation of receptors at a different location in the human body compared to the site where

the drug is intended to work. For example, the muscarinic acetylcholine receptor antagonist scopolamine is used for the treatment of nausea and vomiting due to motion sickness. Side-effects resulting from antagonizing muscarinic acetylcholine receptors in the brain are the anti-cognitive effects such as reduced attention and memory resembling the deficits seen in Alzheimer's disease[27].

Since scopolamine is used as a muscarinic acetylcholine challenge model to induce Alzheimer-like, yet temporary, cognitive effect in healthy subjects[27], CHDR has developed and validated a pharmacological challenge model with mecamylamine. Mecamylamine is a non-competitive nicotinic acetylcholine receptor antagonist[28] which results in cholinergic dysfunction, *i.e.*, transient cognitive deficits, in healthy young males[29,30].

The above mentioned computerized test battery has been used to investigate the potential of different dose levels of mecamylamine to show effects on the neurophysiological and cognitive tests. The expectation was that with increasing dose, the effects on the different tests would also increase. Subsequently these effects were related to the plasma concentrations of the drug, resulting in the plasma concentration–effects (pharmacokinetic–pharmacodynamic) relationship of mecamylamine[29].

The tests incorporated in the design of this study were chosen based on the expected effects known from the literature and the pharmacological mechanism of action of mecamylamine on the nicotinic receptors:

Adaptive Tracking Test:

This is a pursuit-tracking task, for the measurement of visuomotor coordination and sustained attention. A circle of known dimensions moves randomly about a screen. The subject must try to keep a dot inside the moving circle by operating a joystick. If this effort is successful, the speed of the moving circle increases. Conversely, the velocity decreases if the test subject cannot maintain the dot inside the circle. In contrast to non-adaptive tracking methods, this leads to a constant and individually adapted challenge throughout the procedure. The adaptive tracking test used was developed by Hobbs & Strutt, according to specifications of Borland and Nicholson[11].

Smooth Pursuit and Saccadic Eye Movement Tests:

The use of a computer for measurement of saccadic eye movements and smooth pursuit was originally described by Baloh *et al.*[7], and for smooth pursuit by Bittencourt *et al.*[8], and has been extensively validated at CHDR by Van Steveninck *et al.*[19-21] The subject is required to follow a light source with the eyes, which moves horizontally on a screen at 58 cm distance. The light source moves continuously for measurement of smooth pursuit and jumps from side to side for measurement of saccadic eye movements.

VASs:

Assessment of subjective feelings of alertness, mood, and calmness was performed using a set of 16 visual analog lines as described by Norris (1971) and Bond and Lader[10]. Visual analog scores rely on the ability of subjects to semi-quantify a subjective state. Visual analog lines consist of 10-cm line segments. The subject is presented with 16 lines, 1 at a time, on the computer screen. At the two ends of the line, two opposing words representing states of mind (*e.g.*, happy – sad, tense – relaxed) are presented. Subjects put a mark on a point on the line that best represents their subjective state corresponding to the condition tested. The result is a distance (mm) calculated from the mark on the line.

Body Sway:

A string originating from a potentiometer, which is incorporated into the test battery computer, is used to measure postural stability in a single plane while the subject stands still with the eyes closed (described in de Haas *et al*.[12]).

VVLT:

The VVLT is a word learning and memory test, described in more detail in de Haas *et al.*[12] Subjects are presented with a series of 30 words, one by one on the computer screen. The words need to be pronounced and remembered. There are three immediate recall trials, one delayed free recall trial (*i.e.*, without presentation of the words) after approximately 20 min and a recognition trial.

Pharmaco-EEG:

For the standard pharmaco-EEG, electrodes are limited to the midsagittal leads (*Fz*, *Cz*, *Pz* and *Oz*), two electrodes for recording eye movements (outer canthi), and a ground electrode placed 2 cm above the nasion. Changes in the amplitude of the following frequency bands are quantified by spectrum-analysis (*i.e.*, fast Fourier transformation): ß-band (13.5–35 Hz), γ-band (35–48.9 Hz), α-band (7.5–13.5 Hz), and θ-and δ-bands (7.5 Hz or less).

PROTOCOL:

Each independent study using this test battery was approved by independent ethics committees, namely either the 'medical ethics committee of the Leiden University Medical Centre', Leiden, the Netherlands, or the 'Stichting Beoordeling Ethiek Biomedisch Onderzoek, Assen, the Netherlands.

1. Computerized Test Battery Assessments

Note: The test battery should be implemented under controlled conditions (*e.g.*, light intensity, room temperature, and background noise) to minimize influence of exogenous factors on the subject's results. Tests that can be repeatedly performed should be administered at least once before drug administration to serve as baseline. The **Table of Materials** provides an overview of the materials and equipment of the test battery.

1.1. Adaptive tracking test

1.1.1. Switch on the power of the test battery computer and turn on the computer and screens.

1.1.2. Seat the subject in front of the (subject) computer screen and joystick.

1.1.3. Check which is the preferred hand of the subject and adjust the joystick accordingly.

1.1.4. Instruct the subject to hold the joystick as a pen, with the arm resting on the table.

1.1.5. Start the test script via the installed program.

1.1.6. Fill out the requested specifics such as subject and study number.

1.1.7. Execute the test by clicking 'start' on the test assistant screen.

1.1.8. Monitor the performance of the subject on the test assistant screen and encourage the subject to keep the circle around the dot if the subject cannot exceed difficulty factor 2.

1.2. Saccadic eye movement and smooth pursuit test

Note: The eye movement electrodes should be attached to the sites specified in the clinical study protocol based upon the 10-20 System of the International Federation of Societies for Electroencephalography and Clinical Neurophysiology.

1.2.1. Identify the outer canthus of the right eye (*i.e.*, the angle at the outer end of the fissure between the eyelids).

1.2.2. Repeat this procedure for the left eye.

1.2.3. Identify the place for the ground electrode 2 cm above the nasion (*i.e.*, the root of the nose).

1.2.4. Thoroughly rub the sites of the eye electrodes using a cotton-wipe skin cleansing gel for bioelectrical measurement (see step 3.1) to decrease the skin impedance, and use a cotton-wisp stick.

1.2.5. Be careful not to abrade the skin, but do not rub too softly. Wipe away the residual gel with a gauze.

1.2.6. Apply the three self-adhesive electrodes at the prepared sites.

1.2.7. Connect the wires to the eye electrodes. Put your hand behind the press-button of the

electrode to prevent it from pushing into the skin.

1.2.8. Direct the wires along the ears over the shoulder of the subject to prevent the wires from hanging before the eyes.

1.2.9. Plug the three wires in the electrode impedance meter.

1.2.10. Check the impedance on the display: if the impedance is over 5 kΩ, check the quality of the electrode-attachment.

1.2.11. Connect the subject to the eye movement measurement system by plugging all electrodes into the telefector and connect its cable to the amplifier.

1.2.12. Instruct the subject to place the head on the headrest and relax, to follow the light on the screen by moving the eyes, and to not move the head.

1.2.13. Start the test script via the installed program. Fill out the requested specifics such as subject and study number.

1.2.14. Start the test by pressing the spacebar upon the 'go' instruction on the test assistant screen.

1.3. Bond and Lader VAS

1.3.1. Instruct the subject to score how they are currently feeling by using the mouse to mark the visual analog line presented on the screen.

1.3.2. Instruct the subject that the most extreme points on the line represent the most extreme sensation imaginable.

1.3.3. Start the test script via the installed program. Fill out the requested specifics such as subject and study number.

1.3.4. Instruct the subject to start the test by clicking the mouse.

1.4. Body sway

Note: Subjects should wear flat shoes during this test. No instructions or other stimuli are presented on the computer screen.

1.4.1. Ask the subject to stand in front of the computer, with a distance between the feet of about 10 cm, and arms hanging alongside the body.

1.4.2. Attach the string that originates from the potentiometer built into the test battery

computer onto the waist of the volunteer (*e.g.*, the belt, or pants) by using the clip at the end of the string.

1.4.3. Adjust the height of the table with the computer on it until the string is horizontal; a maximum deviation of 5 ° is acceptable. Ask the subject to close his or her eyes.

1.4.4. Start the test script via the installed program. Fill out the requested specifics such as subject and study number.

1.4.5. Start the test by clicking on 'Start Body Sway Sampling Session' on the test assistant's computer screen.

1.5. VVLT

Note: Volunteers are not allowed to write down words at any time during the whole test procedure.

1.5.1. Instruct the subject that during the following automatic (visual) presentation of the words, the subject should name the words when they appear and remember them, and that at the end of the list, all words that are recalled should be named, each word only once.

1.5.2. Start the test script via the installed program. Fill out the requested specifics such as subject and study number.

1.5.3. Instruct the subject to read the written instructions displayed on the screen.

1.5.4. Tell the subject that the test will start when the subject presses the spacebar.

1.5.5. Record the recalled words (correct, incorrect, and words mentioned multiple times) by clicking on the recalled words on the test assistant screen.

1.6. Pharmaco-EEG

Note: The electrodes should be attached to the sites specified in the protocol, and locations are based on the 10-20 System of the International Federation of Societies for Electroencephalography and Clinical Neurophysiology.

1.6.1. Measure and identify the exact location of the electrodes on the subject's head.

1.6.2. Thoroughly rub the site using a cotton-wipe stick and skin cleansing gel to decrease the skin impedance. Be careful not to abrade the skin, but do not rub too softly.

1.6.3. Stand behind the subject and attach the electrodes to the cleansed sites. Work from behind to the front.

1.6.4. Put the cap of the electrode through the box with paste and wipe away the remainder by striking the cap along the brim of the box.

Note: The cap should filled completely, but not overloaded with paste.

1.6.5. Press the electrode on the cleansed site by spreading scalp hair if necessary. Push the electrode on the skin and be careful that as little hairs as possible are under the electrode.

1.6.6. Put the wire of the electrode over the shoulder of the subject into the subject's lap.

1.6.7. Use a small piece of hair to fix the electrode with the paste (which appears from the opening of the electrode cap), and an additional piece of hair (at a right angle to the other piece) with some paste to further fix the electrode to the skin.

1.6.8. Check whether the electrode impedances are below 5 kΩ and adjust if necessary.

1.6.9. Use tape to bundle the wires and to fixate the bundle to the clothes of the subject.

1.6.10. Attach the electrode wires to the recording equipment.

1.6.11. Open the EEG program on the computer.

1.6.12. Instruct the subject to relax and to not move or speak for the measurement period.

1.6.13. Instruct the subject to close the subject's eyes.

1.6.14. Start the test script via the installed program.

[Insert Table 1 here]

REPRESENTATIVE RESULTS:

The computerized test battery assessments generate standardized and electronic data files. See **Table 1** for the specifics on outcome values per test.

The test battery is primarily used in early phase clinical drug studies investigating effects of novel compounds in comparison to a (non-active) placebo or (active) comparator drug. Therefore, the factor 'treatment' should be considered in the statistical analysis of the data. A pre-dose (*i.e.*, drug-free) assessment should be performed for the majority of the tests used in the protocol, to serve as baseline data. The VVLT can only be performed at one time-point post-dose (often at the time-point where the concentration of drug is highest), without pre-dose measurement, the learning effects and the interference of the learning process for the pre-dose and post-dose different word lists are used. As most tests are performed multiple times following drug administration to characterize the time-profile of the drug effects, the effect of time should be

considered in the statistical analysis of the data.

In the protocol here, the test results were analyzed with a mixed model analysis of covariance (ANCOVA) with subject, subject by treatment, and subject by time as random effects; and treatment, study period, and treatment by time as fixed effects. The average baseline value per test was taken as covariate, as baseline measurements were performed twice to prevent loss of baseline data if one of the assessments proved insufficient. Before implementing the mixed model, the data were inspected for normality of distribution by means of Q-Q plots. If necessary, data would be log-transformed to ensure normal distribution.
The analysis is done using the least squared means (LSM) approach, where, per treatment in the analysis an estimate of the mean is calculated by the model (*i.e.*, the LSM). The LSM is not the same as the raw data average for the treatment, because a correction for baseline took place, and missing values were estimated by the model and included in the analysis.

The analysis is presented in LSM graphs, which are based on the estimates of the analysis and are different from average graphs based on the raw data time profile. As LSMs do not have standard deviations, the graphs are made with 95% confidence interval error bars. To avoid overcrowding the graph, only the error bars of the treatment with the highest value are shown up and of the treatment with the lowest value are shown down.

The acute pharmacodynamic effects of a single oral dose of mecamylamine hydrochloride at 10 mg and 20 mg, a 15-min infusion of 0.5 mg scopolamine hydrobromide, and double placebo (oral and intravenous) are shown in **Figure 1** (change from baseline LSM graph). As the VVLT is only performed once post-dose, the VVLT data are shown in a traditional box-plot fashion, with different boxes per treatment (see **Figure 2**).

The protocol described in this paper is part of a larger study described in published literature[29,30] and an in press published paper. The results described below are an example of the results of two computerized battery tests, in 12 healthy young male subjects, in a four-way cross-over design. For further details on the study, please see Baakman *et al.*[30]

As expected, the performance on the adaptive tracking test (the percentage correctly tracked) was negatively influenced by the administration of the cholinergic antagonists mecamylamine and scopolamine. Both the mecamylamine 20 mg and the 0.5 mg scopolamine treatments significantly worsened the score compared to placebo administration. The overall treatment effect was $F = (3,33)\ 43.25$, $p < 0.0001$, the mecamylamine 20 mg estimated difference was -2.06% correctly tracked (95% confidence interval [CI]: -3.97, -0.15) with a $p = 0.0355$ and the scopolamine estimated difference was -10.4% correctly tracked (95% confidence interval [CI]: -12.4, -8.39) with $p < 0.0001$.

When looking at the VVLT, administered once post-dose at +3.5 h for the immediate recall trials and +5 h for the delayed and recognition trials, all treatments induced a poorer performance (*i.e.*, less words remembered) on the third trial of immediate recall and the delayed recall trial (overall treatment effect was $F = (3,33)\ 15.17$, $p < 0.0001$ for the third immediate recall trial and $F = (3,34)$

9.98, $p < 0.0001$ for the delayed recall trial). The two dose levels of mecamylamine showed a dose related effect in that the 20 mg dose showed a larger decrease in total number correctly recalled compared to placebo than did the 10 mg dose compared to placebo. For the third immediate recall trial, the results are: on average -2.7 words (95% confidence interval [CI]: -5.1, -0.3), $p = 0.0286$ for the 10 mg mecamylamine administration, and on average -3.6 words (95% CI: -5.9, -1.4), $p = 0.0025$ for the 20 mg mecamylamine administration. For the delayed recall trial, the results are: on average -3.1 words (95% confidence interval [CI]: -5.8, -0.4), $p = 0.0259$ for the 10 mg mecamylamine administration, and on average -3.8 words (95% confidence interval [CI]: -6.4, -1.2), $p = 0.0051$ for the 20 mg mecamylamine administration. Administration of scopolamine 0.5 mg showed even stronger negative effects on word recall: on average -7.7 words (95% confidence interval [CI]: -10.1, -5.4), $p < 0.0001$ for the third immediate recall trial and on average -7.1 words (95% confidence interval [CI]: -9.8, -4.5), $p < 0.0001$ for the delayed recall trial, all compared to placebo.

Administration of scopolamine in healthy subjects is known to induce large negative effects on cognitive tests results, as was for example described in a large study in 90 healthy male subjects[6]. The above described results show that the tests of the computerized battery were also able to show this significant anti-cognitive effect of 0.5 mg intravenously administered scopolamine. Regarding administration of mecamylamine, literature reports that lower doses of up to 20 mg induce negative effects on cognitive test results[31-33], even though the actual effect is much smaller compared to the effect of scopolamine[30], which is also evident from the results in this protocol.

These results show that the tests from the computerized test battery are sensitive to show acute pharmacodynamic effects after single administrations of the investigated anti-cholinergic drugs. The tests can differentiate between administration of placebo and drug, and more importantly, can differentiate between the muscarinic antagonist scopolamine and the nicotinic antagonist mecamylamine. These effects are repeatedly shown in multiple tests, evident from the statistical results and the similar graphs with tests results (data presented in Baakman *et al.*[30]).

[Place Figures 1 and 2 here]

FIGURE AND TABLE LEGENDS:

Figure 1: Effect of placebo, oral 10 mg and 20 mg mecamylamine, and intravenous 0.5 mg scopolamine on the adaptive tracking test in 12 healthy young males. Time course of mean values (and SD for highest and lowest scores) for the adaptive tracking test, measured at multiple time-points following drug administration (at t = 0), change from baseline data for 12 healthy male subjects. The percentage of correctly tracked is presented on the y-axis, time-point post-dose is presented on x-axis, with double placebo (oral and intravenous) results (grey circle), 10 mg mecamylamine results (magenta square), 20 mg mecamylamine results (green triangles) and 0.5 mg scopolamine (blue diamonds). This figure has been modified from Baakman *et al.*[30]

Figure 2: Effect of placebo, oral 10 mg and 20 mg mecamylamine, and intravenous 0.5 mg scopolamine on the visual verbal learning test in 12 healthy young males. Boxplot results of the

VVLT delayed recognition trial (figure on the left) and third immediate recall trial, with the number of correctly remembered words on the y-axis and treatment on the x axis, for 12 healthy male subjects. The overall treatment effect is shown in the left bottom corner, the *p*-values of individual contrasts of treatment compared to placebo are depicted by means on the asterisks (*). The median is represented by the thick black line in the box. The mean is represented by the red 'M'. The grey circles represent actual data points (*i.e.*, observations). This figure has been modified from Baakman *et al.*[30]

Table 1: Description and specifics of the assessments. Description of the specifics of the individual tests, including a description of the domain that is tested, the administration time, and specific outcome variables.

DISCUSSION:

Proof of pharmacodynamic effect is key in early phase drug development, as it warrants the next step of introducing a new drug in larger numbers of patients[34]. In the case of drugs developed to be active in the CNS it is especially important to show effects which indicate penetration of the blood-brain barrier[35]. Even though a lumbar puncture after a subject has received the drug is often chosen as a proxy for blood-brain barrier penetration, it is an invasive and burdensome technique and moreover, presence of the drug in the cerebrospinal fluid (CSF) does not equal activation of the drug by binding to its target(s) in the brain.

Phase I studies traditionally are data-intensive studies, with multiple series of assessments in close succession, to characterize the pharmacokinetic and pharmacodynamic profile of a new drug. Drugs that work in the CNS are likely to affect more than one neuropsychological and/or neurophysiological domain, as different receptors are often not just located in a single brain region. The main nicotinic receptors involved in cognition are located in the prefrontal, motor, and entorhinal cortices, and with lower density, in the cingulate and temporal cortex, thalamus and basal ganglia[36]. In addition, a single brain region is often connected to multiple other brain regions[37].

Therefore, the computerized test battery core consists of a set of sensitive tests, of which the composition can be altered (*i.e.*, tests can be added or removed from the battery) based on the expected CNS effects, to maximize the chance of positive results. This flexibility allows the battery to be suitable for use in studies with different types of drugs, but also in different populations. For example, in a study investigating a new drug in a small group of 24 patients with Huntington's disease (a neurodegenerative movement disorder), the core test battery was updated to include a test of fine motor skill (the finger tapping test, where in 5 consecutive trials of 10 s each, the spacebar needs to be tapped with the index finger of the dominant hand as quickly as possible), as one of the hallmarks of Huntington's disease are disturbances in fine motor skill[38]. Measurement of fine motor skills is not included in the core test battery, but is of importance to study potential changes in motor functioning in Huntington's disease. Nonetheless, the core tests have remained fairly stable over time, indicating the sensitivity of the battery for effects of a large number of drugs.

The number of tests in the battery should be kept concise to allow for multiple testing following drug administration, where test sessions should be planned such that the (presumed) pharmacokinetic profile of a drug is closely followed. This will result in information on pharmacodynamic effect coinciding with pharmacokinetic processes such as absorption, peak concentration, and elimination of the drug, information that could be combined in a pharmacokinetic-pharmacodynamic model, which was also developed for the protocol described in this paper[29].

In some cases, the exact mechanism of action of an investigational compound is not yet fully understood from studies in animals. Over the past two decades the core tests from the computerized battery have been used to characterize the profile of effects of a large number of different investigational but also registered drugs from which the mechanism of action is known. This has resulted in a database of drug specific profiles, where for different drugs with the same mechanism of action, comparable test battery profiles are observed[3]. This allows for the profile of a new drug to be compared to the profiles of compounds of which the mechanism of action is known, and if a resemblance is found this could give insight into the mechanism of action of the investigational compound. The fact that comparable test profiles have been identified for different compounds with a similar mechanism of action provides strong proof for the sensitivity of the core tests of the test battery for CNS drug effects.

The potential for repeatability over a short period of time following drug administration is vital for the success of a battery like the computerized test battery described in this paper. The CNS is however influenced by both endogenous and exogenous factors, thereby altering a subject's test performance[39]. This highlights the importance of standardization of the conditions of the test environment, together with other subject specific factors. The exact conditions to be maintained during the execution of the tests should be specified in the study protocol and uniformly upheld in all subjects throughout the study. The lighting and room temperature should be kept constant over the testing period and the amount of distraction (noise, multiple persons in the room during testing, *etc.*) should be kept to a minimum. Other factors that could be controlled are certain aspects of the lifestyle of the subjects, such as diurnal rhythm, rest and fatigue, the intake of certain type of food and beverages, and the use of psychoactive substances.

Also, it is a known fact that neuropsychological test outcomes could be influenced by practice, or learning effects[40], especially memory tests such as story and word list learning[41] (*e.g.*, VVLT test). Therefore, specific attention should be on allocated to the number of training sessions and test execution.

Other standardized, computerized test batteries have been developed and are widely used in drug development, with those described in Egerhazi *et al.*[22] and Underwood *et al.*[23] being among the most used in clinical trials. As mentioned before, the computerized test battery described in the current paper is different from these systems in that it also includes measurements of neurophysiological assessments (*e.g.*, pupillometry, eye movement, EEG) by means of easy add-ons to the computer system, in addition to the more traditional neuropsychological tests such as the n-back test (described in Alvarez-Jimenez *et al.*[29]). The other systems however are portable

computers, which makes testing at multiple sites feasible. Currently the set-up of the computerized test battery developed by CHDR is not suited for easy transportation between sites. A more portable version (*i.e.*, laptop) has been designed and is currently being validated. This would allow for testing in multicenter clinical trials and possibly even at the home of, for example, patients who are unable to visit to the research institute due to mobility problems.

The computerized battery is a flexible battery, in the sense that other neuropsychological or physiological tests that have been shown to be sensitive to CNS drug effects can be incorporated in the system. Event related potentials (ERPs)[42] are a recent example of this process: ERPs are gaining interest in clinical research and the demand for the inclusion of tests measuring different ERPs in clinical trials is growing. Ongoing validation of ERPs for implementation into the computerized test battery is currently being performed at CHDR.

In summary, the standardized, computerized test battery of neuropsychological and neurophysiological assessments described in this paper is designed to investigate pharmacodynamic effects of CNS active drugs in early phase drug development. The core tests have reliably and repeatedly shown to be sensitive to CNS effects, indicating penetration of the blood brain barrier and pharmacological activation of target sites in the CNS.

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The authors have nothing to disclose.

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Figure 1

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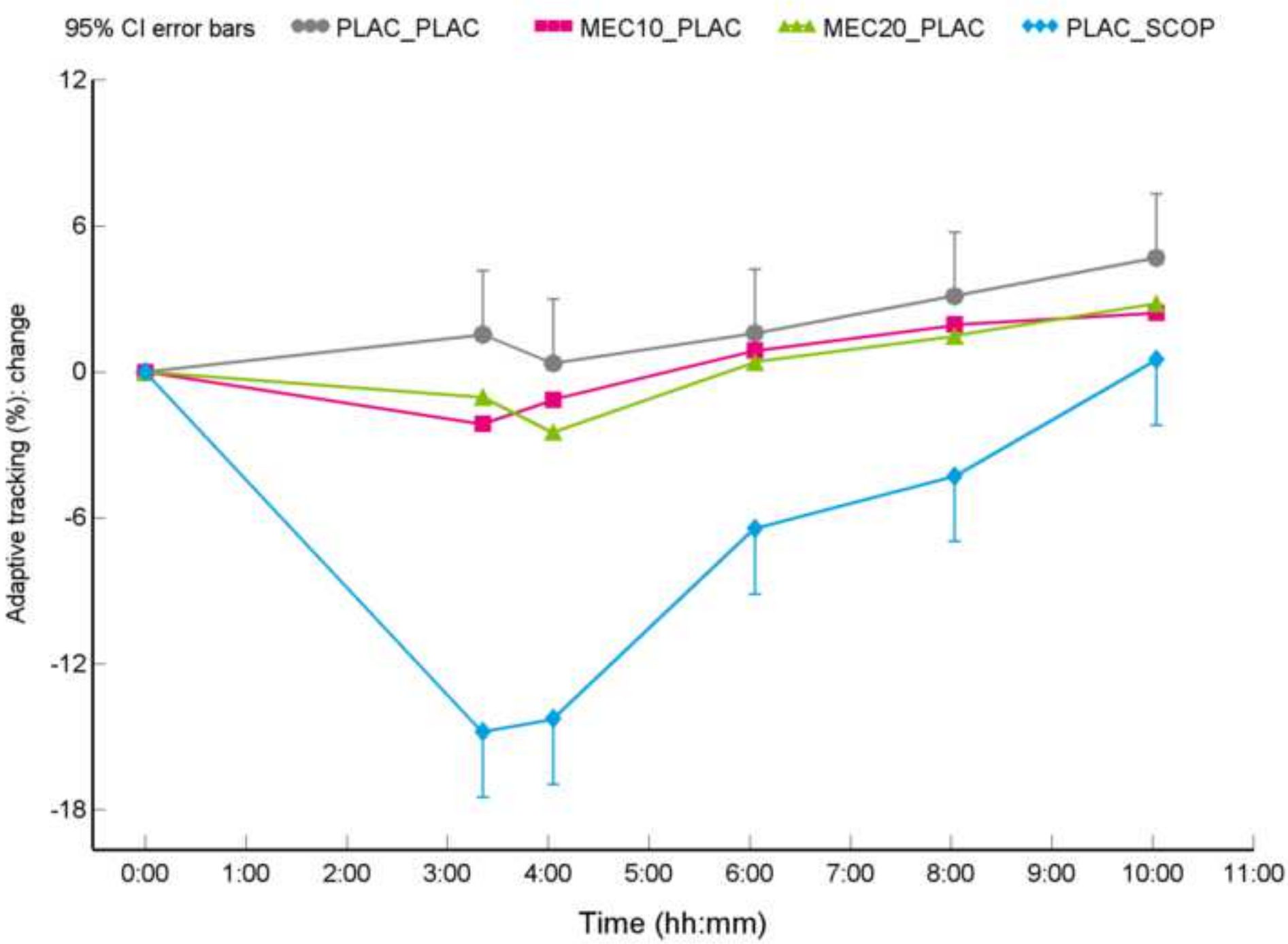

Figure 2A

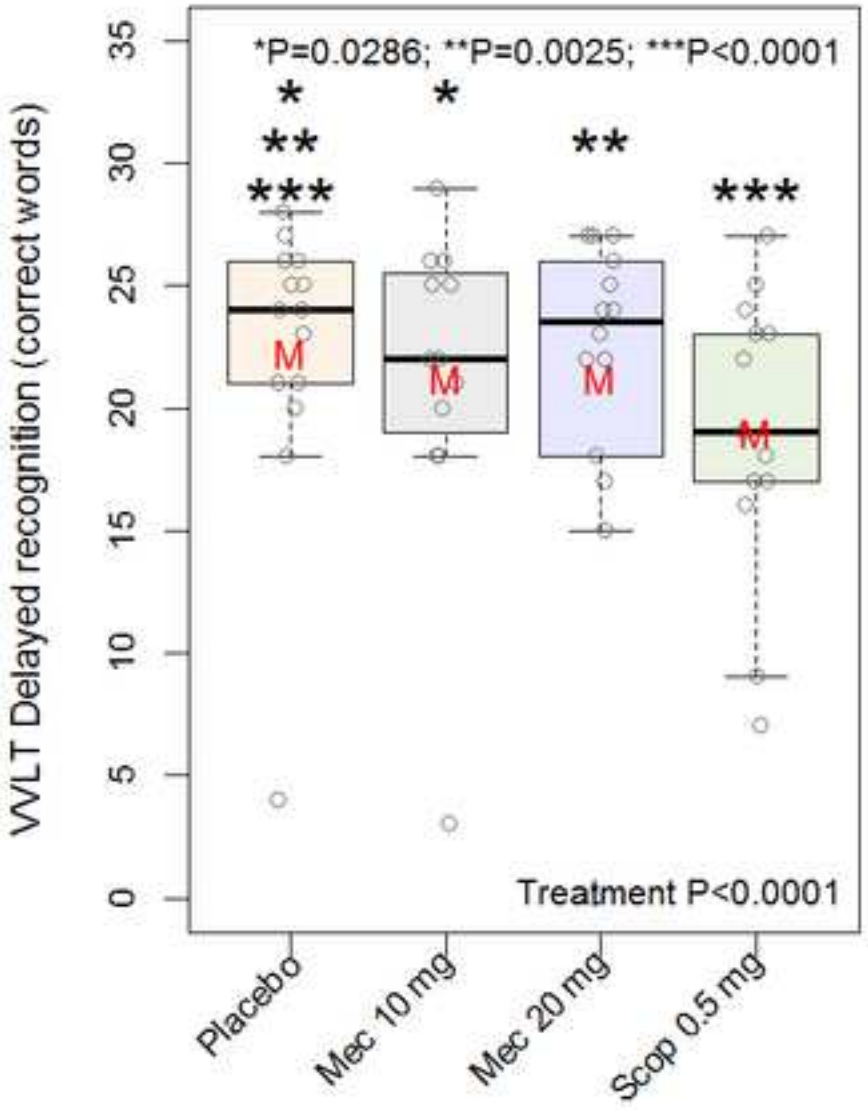

Figure 2B

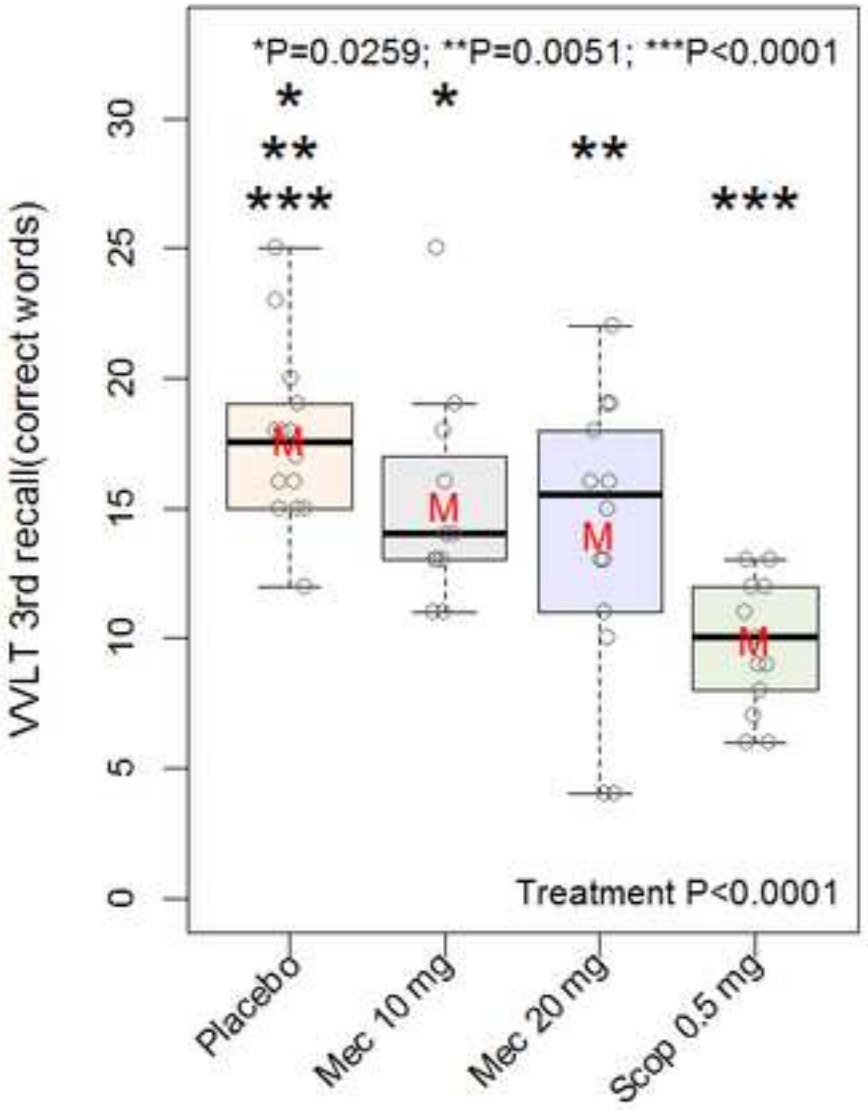

Table 1

Assessment	Domain
Adaptive tracking test	Visuo-motor coordination, vigilance
Saccadic eye movement test	Saccadic eye movements
Smooth pursuit test	Smooth pursuit
Body sway test	Postural control in a single plane
Visual analogue scales (B&L)	Subjective assessment of alertness, mood, calmness
Visual verbal learning test	Learning, short- and long term memory, retrieval

Pharmaco-EEG	quantitative, cerebral EEG-activity

Description

A circle moves randomly about the computer screen. The subject must try to keep a dot inside the moving circle by operating a joystick. If this effort is successful, the speed of the moving circle increases. The velocity is reduced if the test subject cannot maintain the dot inside the circle.

The subject is required to follow a light source with only the eyes, which moves horizontally on a screen at 58 cm distance. The light source jumps from side to side for measurement of saccadic eye movements.
The subject is required to follow a light source with only the eyes, which moves horizontally on a screen at 58 cm distance. The light source moves continuously for measurement of smooth pursuit.
The subject is asked to stand still, with eyes closed while attached to the meter by means of a cord. The feet should be approximately 10 cm part and the hands in a relaxed position alongside the body.

Subjects are asked to indicate how they feel concerning a specific state by clicking on a line of 100 mm, flanked by two opposite adjectives (e.g. drowsy - awake). The test consists of 16 items (i.e. lines).

Subjects are presented 30 words in three consecutive word trials, i.e. word learning test. Each trial ends with a free recall of the presented words (Immediate Recall- a test to determine acquisition and consolidation of information). Approximately 30 minutes after start of the first trial, the subject is asked to recall as many words as possible (Delayed Recall- this test measures active retrieval from long term memory). Immediately thereafter, the subject undergoes a memory recognition test, which consists of 15 presented words and 15 ‘distractors’ (Delayed Recognition- testing memory storage).

Subjects are asked to relax and depending on the protocol keep their eyes open or closed.

Outcome values	Specifics
Percentage of time correctly tracked	Administration time: 4 minutes
Percentage of time the subject's eyes are in smooth pursuit of the target, for each stimulus velocity and for each stimulus frequency	Administration time: 2 minutes.
Peak velocity (deg/s), reaction time (s), jump size (deg), primary saccadic deflection (deg) and inaccuracy (%) are calculated for each saccadic eye movement	Administration time: 2 minutes.
Antero-posterior movement in mm	Administration time: 2 minutes.
All scores are measured in mm, from the beginning of the line on the left side to the point where the mark produced by the subject crosses the line. The score represents the adjective on the right side of the line (*e.g.* a higher score on a scale marked awake - drowsy indicates that the subject feels drowsier). Composite scores for the three domains are computed: the composite score for alertness is composed of nine scores, mood of five, and calmness of two.	Administration time: 2 minutes.
Per trial a total number correct, total number incorrect and total number of doubles are recorded. For the recognition trial, total number correct, total number incorrect and reaction time (and SD of RT) are recorded.	Administration time: 10 minutes

For each lead (frontal lead: **frontal** (Fz) - central (Cz), **central** lead: Cz - parietal (Pz), **parietal** lead: Pz - occipital (Oz)), fast Fourier transformation analysis is performed to obtain the sum of amplitudes in the delta- (2-4 Hz), theta (4-7.5 Hz), alpha- (7.5-13.5 Hz), beta- (13.5-35 Hz), and gamma-(35-48.9 Hz) frequency ranges	Administration time: 4 minutes

Table of Materials/Equipment

Click here to download Materials Table JoVE_Table 2_Materials_Hart.xlsx

Name of Material/ Equipment
NeuroCart general computer hardware
Amplicon Impact E70 (=computer)
Medical insulation transformer
24 inch widescreen
PS2 Mouse
PS2 Keyboard
Photocamera
EOS utility program
17 inch computer screen (research assistant)
USB keyboard (research assistant)
USB mouse (research assistant)
NeuroCart general computer software
Windows 7 or higher
E-prime 2.0
EEG and eye electrodes hardware
Grass series Amplifier Systems
Quad, wide-band, high-gain, programmable AC amplifier
Quad, high-gain, programmable AD amplifier
Bioelectric Input Box, Electrode Board Model BIPOLA
Electrode Impedance Meter
A/ D converter
Gold electrodes
Ambu ECG electrodes
EC2 cream
Nuprep
EEG and eye electrodes software
Grass link 15 software
Spike 2
Adaptive tracking materials (hard and software)
Adaptive tracking joystick
TrackerUSB
Bodysway hardware
Posturograph

Medical insulation transformer
Grass series Amplifier Systems
Quad, wide-band, high-gain, programmable AC amplifier
Quad, high-gain, programmable AD amplifier
Bioelectric Input Box, Electrode Board Model BIPOLA

Company
Thalheimer Trenntransformator
DELL
DELL
DELL
Canon
Canon
DELL
DELL
DELL
Microsoft
Psychology Software Tools, Inc. (PST)
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus
Cambridge Electronic Design (CED), Cambridge, UK
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus
BlueSensor
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus
Weaver and Company
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus
Cambridge Electronic Design Limited
Job Kneppers Ontwerp en Realisatie B.V., Delft.
Kevin Hobbs, CarbisDesign, UK
Sentech BV

Thalheimer Trenntransformator
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus
Grass-Telefactor, An Astro-Med, Inc. Product Group/Natus

Catalog Number	Comments/Description
ERT 230/23/6G	
U2412M	for subject
	for subject
	for subject
EOS 1100D	
N.A.	photocamera software
1708FP monitor	for research assistant
	for research assistant
	for research assistant
N.A.	every test has a custom, internally valida For VVLT, VAS
15LT	amplifier for EEG electrodes
15A54	part of the 15LT ampyfier
15A94	
15LT	input box for electrodes
F-EZM5	
1401 Mk1 and Mk2	
Fx-E5GH	EEG electrodes
N-OO-s/25	Eye electrodes
N.A.	electrode cream
N.A.	Skin prep gel
N.A.	
N.A.	every test has a custom, validated script For bodysway, eye movements a
N.A.	custom built
N.A.	Adaptive tracking software
Celesco SP2 -50	

ERT 230/23/6G
15LT
15A54
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CORRESPONDING AUTHOR:

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Department: CNS

Institution: Centre for Human Drug Research

Article Title: The NeuroCart: studying pharmacodynamic effects of chol.... development

Signature: 

Date: 04 May 2017

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Response to reviewers - JoVE56569

Changes recommended by the JoVE Scientific Review Editor:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
We have done this.

• Some sections of the representative results show significant overlap with previously published work. Please re-write the text highlighted in red font in the attached document to avoid this overlap.
We have rewritten the indicated section. The overlap with previously published work is probably work on the PainCart (J.L. Hay et al.), also developed by the Centre for Human Drug Research: the exact similar method of analyzing is applicable to the NeuroCart and the PainCart data.

• **Abstracts:**
1) Please revise the Short Abstract so that it clearly states the goal of the protocol within 50 words.
The short abstract has been re-written to meet the suggestions.

• Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the guidelines of your institutions human research ethics committee.
The statement regarding ethical approval for this protocol is updated according to the reviewer's comments.

• **Protocol Language:** The JoVE protocol should be almost entirely composed of numbered short steps (2-3 related actions each) written in the imperative tense (as if you are telling someone how to do the technique, i.e. "Do this", "Measure that" etc.). Any text that cannot be written in the imperative tense may be added as a brief "Note" at the end of the step (please limit notes).
1) Please move the descriptive sections of the protocol to introduction, Representative Results or Discussion. The JoVE protocol should be a set of instructions rather a report of a study. Any reporting should be moved into the representative results.
The descriptive section in the protocol section have been moved to the introduction section.
2) Line 143-164: Please merge these and condense into a single note of no more than ~10 lines.
This section has been rewritten in the introduction section.
3) Please edit the protocol so that ALL steps are in the imperative tense. Example not in imperative tense: "The subject is instructed to score how they are currently feeling." This can be rewritten as "Instruct the subject to score how they are currently feeling."
The steps have been rewritten zo that they are now stated in the imperative sense.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more details to the following protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure that all additional details in the protocol section are written in the imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.).
1) 1.2.5: Are there 3 electrodes in total? Please mention where the electrodes are applied.
There are indeed 3 electrodes in total, 2 eye electrodes and 1 ground electrodes. The sites for the application of the sites is provided in the preceding steps. We have clarified that there are 3 electrodes in

this step.
2) 1.2.12: How? Do you simply push a button?
A computer program (script) must be started by the research assistant. This has been clarified and added in the protocol steps.
3) 1.3.1: It is unclear what is done here. What is the score scale used here?
In the introduction we have added information on this test with examples of the visual analogue scale lines, so that it is more clear what is happening. Also, the steps in the protocol have been updated.
4) 1.3.3: How? What is happening in this test? Does something happen on the screen? Please add a note to describe what the subject sees.
In the introduction we have added information on this test with examples of the visual analogue scale lines, so that it is more clear what is happening. Also, the steps in the protocol have been updated.
5) 1.4.4: How? What is happening in this test? Does something happen on the screen? Please add a note to describe what the subject sees.
In the introduction we have added information on this test with examples of the visual analogue scale lines, so that it is more clear what is happening. Also, the steps in the protocol have been updated.
6) 1.5.1: This is not clear enough.
In the introduction we have added information on this test with examples of the visual analogue scale lines, so that it is more clear what is happening. Also, the steps in the protocol have been updated.
7) 1.5.2: How? What is happening in this test? Does something happen on the screen? Please add a note to describe what the subject sees.
In the introduction we have added information on this test with examples of the visual analogue scale lines, so that it is more clear what is happening. Also, the steps in the protocol have been updated.
8) 1.6.14: How?
In general: in the introduction we have added some explanation on the different tests, including references so that it is more clear what is happening to the subject and on the screen. Via the references additional information can be accessed, allowing for replication of the protocol. In addition, the above indicated steps have been reviewed and updated with more detailed information.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) Please highlight 1.2.5 for continuity.
2) **Please ensure that the manuscript title best reflects the filmable content (i.e. the portions you highlight).**
The above suggestions have been implemented.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
Where possible, this has been implemented.
A section about troubleshooting is in our view not feasible to describe, the methods described in this paper comprise a rather complex computer set-up and several different tests, for which no short troubleshooting section can be written that would fit the paper. Therefore, troubleshooting has not been described.

• **Figures:**:
1) Fig 1,2 : Please define error bars, mention sample sizes.
The definition of the error bars has been incorporated in the figures (i.e. 95% confidence interval bars, top left of the figure), as well as in the results section text. We have added the sample size in the legend of the figures, not in the figures themselves.
2) Please provide each figure as an individual PDF, TIFF, JPEG or PNG files.
The figures are provided in TIFF or PNG.

• **Figure/Table Legends:**: Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.
The legends of the figures have been expanded and updated. The legend of the tables were erroneously not included in the initial submission and have been added.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are NeuroCart, Cantab, Cogstate, skinPure, AMBU Blue Sensor N, Grass,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript (including the title) with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
The commercial language has been removed and where necessary references have been added in the text.

• **Table of Materials:**Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as software.
The table of Materials has been re-organized and updated (now hard and software per test and for the whole of the NeuroCart is separated for easier reading and understanding).

• Please define all abbreviations at first use.
This has been done.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.
This has been done.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

The figures have been modified from a previous publication by the authors from the current JoVE paper. According to the guideline for permission for reuse from the British Journal for Clinical Pharmacology (where the figures are published) original authors can reuse their materials without explicit permission from the Journal.

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

Hart et al./Groeneveld present the computerized test battery NeuroCart that their group at CHDR has developed over the last 20 years. NeuroCart is designed to characterize pharmacodynamic effects of CNS-targeting drugs, as well as to monitor CNS side effects of peripheral drugs. Compared to other existing test batteries, e.g. Cantab or Cogstate, the NeuroCart battery offers multimodality by combining neurocognitive tests with neurophysiological measurements.The authors' test battery has been validated and published in several peer-reviewed articles. Overall, this is a timely article that will be useful to the clinical Neuroscience community interested in practical applications of early pharmacodynamic readouts and computerized drug-testing batteries.

Major Concerns:

I do not have any major concerns.

Minor Concerns:

- I am confused about the author's interpretation of the data shown in Fig.1 and Fig.2. In lines 331-333 the authors state that "the effects of 10 mg of mecamylamine
administration is comparable to placebo Administration of 20 mg of mecamylamine led to worse performance on the adaptive tracker test in comparison to placebo". By inspecting the plotted mean+/- error values, one would get an impression that both 10 mg and 20 mg mecamylamine are comparable to placebo and indistinguishable from each other; in contrast, scopolamine appears clearly different from placebo (at least at the 3.5h and 4.5h time points in Fig.2, possibly also at 6h and 8h in Fig.1). Since the authors do not provide any p values, it is difficult to understand what their conclusion about the difference between the effects of these two doses of mecamylamine is based on.

We would like to thank the reviewer for their comments and suggestions. We agree that the way it was written it was not easy to interpret the data. The text has been updated to include the statistical data (95% confidence intervals and p-values etc).

- Please spell out PD in the abstract (line 59); the word "pharmacodynamics (PD)" appears in Introduction (line 71), but the reader of the Abstract alone is left to guess whether PD means Parkinson's disease, pharmacodynamics or something else.

This was corrected.

- In line 139, please refer to either the names of the Ethics Committees, or to the publications where this information can be found (presumably, ref.3-8 and ref.11?)

The specific ECs were added.

- Line 319: when mentioning the ANOVA test, please indicate whether the data were tested for normality of their distribution prior to applying this test.

Information on checking for normality has been added.

- Line 337: please provide the p vaule next to the claim that the the results were "small, albeit statistically significant".

The text has been rewritten, also based on other comments, so this particular sentence has been dropped from the text. However, we agree with the reviewer that without information on statistical

ooutcome the results are difficult to interpret on figures alone. Therefore, in the results text the mean differences between treatments, including 95% confidence intervals and p-values have been added.
- Some typos that I spotted:
line 203 - consider replacing "rests of gel" with "the remaining gel";
Your suggestion has been implemented.
line 276 - replace "do not rub to softly" with "do not rub too softly".
This has been corrected.

Reviewer #2:

Manuscript Summary:

The authors describe use of Neurocart, a computerized battery of behavioral and electrophysiological tests, as a means to evaluate whether a drug acts on CNS targets and provide insight into potential mechanisms of action. Specifically, healthy subjects were subjected to temporary, reversible mimicry of disease by application of the anticholinergic drug mecamylamine.

Major Concerns:

Neurocart provides a useful preclinical tool that was developed by the authors and has been used to test several drugs. The title and abstract are appropriate (the abbreviation "PD" is included in the abstract but not defined). For the most part, the text clearly describes Neurocart and its use, although the writing style is heavy on jargon and there are many cases where clarity is an issue (see below). The descriptions of the utility / value of Neurocart are vague.

We would like to thank the reviewer for their comments and suggestions. We agree that the text is heavy in jargon and have implemented the suggestions by the reviewer for specific parts of the introduction and discussion. In addition we have reviewed the whole text and have rewritten parts to avoid jargon and vague use of language.

Minor Concerns:

1. The Figures could be more aesthetic (e.g., larger symbols) and the legends are inadequate. There are several abbreviations that indicate experimental groups but these are not defined anywhere.

We have updated the figures to be more clear. Symbols and abbreviations used are defined in the legend.

2. It is never clearly stated which "electrophysiology" is included in the battery of tests (a short list is provided near the end of the Discussion (lines 377-380). This would be more useful in the introduction).

Specification on electrophysiology tests has been included in the introduction text.

3. There are several vague phrases used without adequate explanation. For example, "domain of CNS", "to be used as a challenge model", "adaptive in nature", "densely measure", and "multimodal in design". In all of these cases, it would be much clearer to just say what is meant rather than invent a phrase that is unclear. So instead of saying "multimodal in design" (lines 103-104) just say the battery includes both behavioral tests and electrophysiological measurements. I am not surewhat "densely measure" means. Rather than "adaptive in nature", just say that you can vary which tests are used to fit each project.

We agree with the reviewer that some jargon has been used. We have re-written large sections of the introduction and discussion with this in mind to avoid unclear phrasing.

4. In many places, references are not included but are needed (e.g., lines 83-94, lines 111-113, lines 390-395, lines 414-416). There is not enough information provided to actually do any of the individual tests. While it would be inappropriate to include all of the methodology here, a few key references that would allow the reader to perform the tests would be helpful.

We agree with the reviewer that there was not enough reference to existing literature. We have added references throughout the manuscript, linking the text more to the published literature.

In addition, we have added a section to the introduction where more explanation on the individual tests is provided, as well as more detailed instruction in the protocol section. Also, references for the tests have been added, so that it more elaborate descriptions on how to perform can be found in the literature as the current paper does not allow for all detail that is needed to perform the tests.

5. Lines 374-382: It would be useful to list all of the tests in Neurocart and then show how the battery can be tailored to specific projects (rather than just say it can be done).
This suggestion has been implemented by describing an example.
6. "PD" is used several times but not defined (line 130, line 323). Is this "post drug"? If so, it does not seem necessary - just say what happens in the presence of the drug.
We have removed the abbreviations from the manuscript and have either stated 'pharmacodynamic' or just 'effect' where appropriate.
7. "MoA" is not a useful abbreviation (lines 381,390). It is only used 3-4 times so it would be better to just write out "mechanism of action".
This suggestion has been implemented.
8. Line 122: Does "Here" refer to this paper, or are the authors defining the pharmacological challenge model (it needs a clear definition)?
This has been rewritten.
9. Lines 402-412: No data are presented in this manuscript to test the reproducibility of Neurocart tests.
In the introduction and in the discussion it is stated several times that the tests have been used in multiple studies with different drug types (including references to these studies), thereby providing evidence of the reproducibility of the test battery.