Acute modulation of the cholinergic system in the mouse brain detected by pharmacological resting-state functional MRI

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ABSTRACT

Introduction: The cholinergic system is involved in learning and memory and is affected in neurodegenerative disorders such as Alzheimer's disease. The possibility of non-invasively detecting alterations of neurotransmitter systems in the mouse brain would greatly improve early diagnosis and treatment strategies. The hypothesis of this study is that acute modulation of the cholinergic system might be reflected as altered functional connectivity (FC) and can be measured using pharmacological resting-state functional MRI (rsfMRI).
Material and methods: Pharmacological rsfMRI was performed on a 9.4 T MRI scanner (Bruker BioSpec, Germany) using a gradient echo EPI sequence. All mice were sedated with medetomidine. C57BL/6 mice ($N = 15$/group) were injected with either saline, the cholinergic antagonist scopolamine, or methyl-scopolamine, after which rsfMRI was acquired. For an additional group ($N = 8$), rsfMRI scans of the same mouse were acquired first at baseline, then after the administration of scopolamine and finally after the additional injection of the cholinergic agonist milameline. Contextual memory was evaluated with the same setup as the pharmacological rsfMRI using the passive avoidance behavior test.
Results: Scopolamine induced a dose-dependent decrease of FC between brain regions involved in memory. Scopolamine-induced FC deficits could be recovered completely by milameline for FC between the hippocampus–thalamus, cingulate–retrosplenial, and visual–retrosplenial cortex. FC between the cingulate–rhinal, cingulate–visual and visual–rhinal cortex could not be completely recovered by milameline. This is consistent with the behavioral outcome, where milameline only partially recovered scopolamine-induced contextual memory deficits. Methyl-scopolamine administered at the same dose as scopolamine did not affect FC in the brain.
Conclusion: The results of the current study are important for future studies in mouse models of neurodegenerative disorders, where pharmacological rsfMRI may possibly be used as a non-invasive read-out tool to detect alterations of neurotransmitter systems induced by pathology or treatment.

Introduction

Impairments of neurotransmitter systems are a common feature of neurodegenerative disorders and occur at early stages of disease progression. These deficits of neurotransmitter systems lead to impaired communication between brain regions and eventually behavior disruptions at later stages (Penzes et al., 2013; van Spronsen and Hoogenraad, 2010). For example, in Alzheimer's disease (AD) it was reported that the toxic effects of amyloid pathology and inflammation induce synaptic defects in the cortex and hippocampus, eventually leading to cognitive dysfunctions (Spires-Jones and Knafo, 2012). The neurotransmitter system that is most severely affected in AD is the cholinergic system. The cholinergic network is important for learning and memory, functions that are affected in AD patients (Schneider et al., 2014). The pathological phenotypes are probably a consequence of a complex interplay of events that develop over time. The existence of mouse models of neurological disorders allows studying how specific aspects of pathology affect neurotransmitter systems. Therefore, a non-invasive tool that enables the detection of modulated neurotransmitter systems in the mouse brain would greatly improve the efficacy of early diagnosis and support the development of new treatment strategies.

Functional magnetic resonance imaging (fMRI) techniques have gained a significant role in improving our understanding of brain function. Resting-state functional MRI (rsfMRI) is used to evaluate functional

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connectivity (FC) in the brain and was first described by Biswal et al. (1995). They showed that stimulation of the motor cortex by hand movement revealed activation, i.e. increased blood oxygenation, of motor areas in the human brain. During rest, the time courses of the blood-oxygenation-level-dependent (BOLD) signal of these motor areas correlated in the lower frequencies (0.01–0.1 Hz). They concluded that this correlation of low frequency fluctuations of blood oxygenation between brain regions during rest could be a manifestation of brain FC. The great advantage of rsfMRI is its non-invasive character and relatively high spatial and temporal resolution. It has been applied to study diseases in the human brain (Buckner et al., 2008; Sheline and Raichle, 2013; van den Heuvel and Hulshoff Pol, 2010) and recently also the healthy (Jonckers et al., 2011; Lu et al., 2012) and diseased (Shah et al., 2013) rodent brain.

Previous studies in human subjects demonstrated that decreasing cholinergic neurotransmission with a cholinergic antagonist affects the BOLD signal in the hippocampus (Antonova et al., 2011; Sperling et al., 2002). AD patients, who display severe deficits of cholinergic synapses and loss of cholinergic neurons (Bales et al., 2006; Pepeu and Giovannini, 2004; Schneider et al., 2014; Watanabe et al., 2009), show altered FC in cortical regions and the hippocampus (Sheline and Raichle, 2013). Furthermore, stimulating the cholinergic system in AD patients increased FC in the cortex (Li et al., 2012) and hippocampus (Pa et al., 2013; Risacher et al., 2013). RsfMRI studies targeting the cholinergic system in rodents are still quite limited. However, an fMRI study in rats showed altered brain activity in frontal brain regions after diminishing cholinergic neurotransmission (Kocsis et al., 2014).

This study aims to detect the effects of acute pharmacological modulations of the cholinergic system on FC in the mouse brain. Because modulating neurotransmission implies that brain function is changed before the rsfMRI acquisition, the term 'resting-state' might not be applicable in this context. Therefore, we refer to this concept as pharmacological rsfMRI. The present study investigates the effects of scopolamine, a muscarinic acetylcholine receptor (mAChR) antagonist, on brain FC in a dose-dependent manner and evaluates whether scopolamine-induced FC deficits can be reversed using a mAChR agonist, milameline. This is the first study showing the ability of pharmacological rsfMRI to detect cholinergic modulations in the mouse brain.

Material and methods

Animals and ethics statement

Male C57BL/6 mice of 12 weeks old were used throughout the whole study (C57BL/6, Jax mice strain, Charles River Laboratories). All procedures were performed in strict accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The protocols were approved by the Committee on Animal Care and Use at the University of Antwerp, Belgium (permit number 2012-48) and all efforts were made to minimize animal suffering.

MRI procedures

For the handling procedures the mice were anesthetized with 2% isoflurane (IsoFlo, Abbott, Illinois, USA), which was administered in a mixture of 70% N_2 and 30% O_2. A subcutaneous (s.c.) catheter was used to administer saline, scopolamine (scopolamine hydrobromide trihydrate, Sigma Aldrich), milameline (milameline hydrochloride, Santa Cruz Biotechnology) or methyl-scopolamine (scopolamine methyl bromide, Sigma Aldrich). The physiological status of all animals was monitored throughout the imaging procedure. A pressure sensitive pad (MR-compatible Small Animal Monitoring and Gating system, SA Instruments, Inc.) was used to monitor breathing rate and a rectal thermistor with feedback controlled warm air circuitry (MR-compatible Small Animal Heating System, SA Instruments, Inc.) was used to

maintain body temperature at (37.0 ± 0.5) °C. During the phMRI and rsfMRI imaging procedures, medetomidine (Domitor, Pfizer, Karlsruhe, Germany) was used to sedate the animals as previously described (Jonckers et al., 2011). After the imaging procedures, the effects of medetomidine were counteracted by injecting of 0.1 mg/kg atipamezole (Antisedan, Pfizer, Karlsruhe, Germany).

MRI procedures were performed on a 9.4 T Biospec MRI system (Bruker BioSpin, Germany) with the Paravision 5.1 software (www.bruker.com). Images were acquired using a standard Bruker cross coil setup with a quadrature volume coil and a quadrature surface coil for mice. Three orthogonal multi-slice Turbo RARE T2-weighted images were acquired to render slice-positioning uniform (repetition time 2000 ms, echo time 15 ms, 16 slices of 0.4 mm). Field maps were acquired for each animal to assess field homogeneity, followed by local shimming, which corrects for the measured inhomogeneity in a rectangular VOI within the brain.

Pharmacological MRI

PhMRI ($N = 8$ mice) was performed to determine in which brain regions scopolamine induces signal intensity changes. PhMRI data were acquired using a T2*-weighted echo planar imaging (EPI) sequence (repetition time 15,000 ms, echo time 25 ms, 16 slices of 0.4 mm, 240 repetitions). The field-of-view was (20×20) mm² and the matrix size was (128×64), resulting in voxel dimensions of (0.156×0.312) mm². Baseline scans were acquired for 15 min (60 repetitions) after which either saline (10 ml/kg, s.c.) or scopolamine (3 mg/kg, s.c.) was administered through a catheter (injected volumes 0.3 ml) and scanning continued until 45 min (180 repetitions) post-injection.

Pharmacological resting-state functional MRI

Resting-state signals were measured using a T2*-weighted single shot EPI sequence (repetition time 2000 ms, echo time 15 ms, 16 slices of 0.4 mm, 150 repetitions). The field-of-view was (20×20) mm² and the matrix size was (128×64), resulting in voxel dimensions of (0.156×0.312) mm². Mice ($N = 15$/group) were injected with either saline (10 ml/kg, s.c.), scopolamine (0.5 mg/kg;1 mg/kg;3 mg/kg, s.c.), milameline (1 mg/kg, s.c.) or methyl-scopolamine (3 mg/kg, s.c.) (injected volumes 0.3 ml). Pharmacological rsfMRI scans were acquired 25 min post-injection to ensure that the pharmacological substances have reached the brain.

Another group of mice ($N = 10$) were subjected to the following protocol: baseline pharmacological rsfMRI scans were acquired, after which scopolamine (1 mg/kg, s.c.) was administered and pharmacological rsfMRI scans were acquired again 25 min later. Then, the mice were injected with milameline (1 mg/kg, s.c.) and pharmacological rsfMRI scans were taken 25 min after the milameline injection (injected volumes 0.3 ml). To take into consideration that the observed FC recovery might be due to scopolamine wash-out instead of milameline administration, an additional group of scopolamine-injected (1 mg/kg, s.c.) mice were subjected to pharmacological rsfMRI scans 60 min after scopolamine injection.

MRI data pre-processing

Pre-processing of the phMRI and rsfMRI data, including realignment, normalization and smoothing, was performed using SPM8 software (Statistical Parametric Mapping, http://www.fil.ion.ucl.ac.uk). First, all images within each session were realigned to the first image. This was done using a least-squares approach and a 6-parameter (rigid body) spatial transformation. Second, all datasets were normalized to a study specific EPI template. The normalization steps consist of a global 12-parameter affine transformation followed by the estimation of the nonlinear deformations. Finally, in plane smoothing was done using a Gaussian kernel with full width at half maximum of twice the voxel size (Jonckers et al., 2011).

MRI data analysis

Pharmacological MRI

The phMRI data analysis was performed in SPM8. For each animal the smoothed data were fitted to a generalized linear model (GLM) in which the motion parameters, resulting from the realignment, were included as covariates to account for movement. For each animal two conditions were compared: the first 60 repetitions (pre-injection) and the last 100 repetitions (20 min post-injection). Mean statistical difference images of the pre-injection vs. post-injection conditions were computed for the saline and scopolamine group in SPM8 and presented by overlaying them on an EPI image.

Pharmacological resting-state functional MRI: ROI-based analysis

The REST toolbox (REST1.7, http://resting-fmri.sourceforge.net) was used to analyze the pharmacological rsfMRI data. The smoothed data and a mask for each ROI (the orbitofrontal cortex, the cingulate cortex, the somatosensory cortex, the thalamus, the hippocampus, the retrosplenial cortex, the rhinal cortex, the auditory cortex, the visual cortex and the ventral tegmental are/substantia nigra) were loaded into the software and the motion parameters resulting from the realignment were included as covariates to correct for possible movement that occurred during the scanning procedure. The filter was set between 0.01 and 0.1 Hz to retain the low frequency fluctuations of the time course of the BOLD-signal that are of interest when performing FC studies. The time courses of the BOLD-signal were extracted for each ROI and correlation coefficients between the time traces of each pair of ROIs were calculated. These correlation coefficients were z-transformed using an in-house program developed in MATLAB (MATLAB R2013a, The MathWorks Inc. Natick, MA, USA) and represented in a correlation matrix. Mean z-transformed FC matrices were calculated for each group of mice i.e. the saline, scopolamine and milameline group.

Pharmacological resting-state functional MRI: seed-based correlation analysis

Using the REST-toolbox, individual z-transformed FC-maps were obtained for all saline, methyl-scopolamine and scopolamine-treated animals with the left hippocampus, cingulate cortex or visual cortex as seed regions. The same was done for all animals of which pharmacological rsfMRI scans were acquired at baseline and after the subsequent administration of scopolamine and milameline. These individual z-transformed FC-maps were loaded in SPM8 and mean zFC-maps were computed per group. The mean zFC-maps for each seed region are presented by overlaying them on the EPI-image.

Then, using the individual hippocampus zFC-maps of each animal, the strength of functional connectivity with the hippocampus, represented as *T*-values, was calculated for the thalamus using a mask for this region. The same was done for the cingulate cortex zFC-maps of each animal which were used to calculate FC strength with the retrosplenial cortex, rhinal cortex and visual cortex. Finally, the visual cortex zFC-maps of each animal were used to calculate FC strength with the retrosplenial cortex and the rhinal cortex. This allowed to analyze 6 functional connections i.e. FC between the hippocampus and thalamus, between the cingulate and retrosplenial cortex, between the cingulate and rhinal cortex, between the cingulate and visual cortex, between the visual and retrosplenial cortex and between the visual and rhinal cortex.

Behavior study

Passive avoidance learning was tested in a step-through box during the dark phase of the animal's activity cycle. The step-through box consisted of a first, brightly lit compartment connected with a second, dark compartment by means of a sliding door. Mice were put in the illuminated compartment, and after 5 s the sliding door connecting both compartments was opened. Upon complete entry into the dark compartment (4-paw criterion), animals received a slight foot shock (0.3 mA, 1 s). Exactly 24 h later, the escape latency to re-enter the dark compartment was timed up to 300 s. The passive avoidance test was performed in four groups of animals: a group treated with saline ($N = 8$, 10 ml/kg, s.c.), a group treated with scopolamine ($N = 8$, 1 mg/kg, s.c.), and a group treated with milameline ($N = 8$, 1 mg/kg, s.c.). Training for the passive avoidance test was performed 55 min after injection for these groups. A fourth group was subsequently treated with scopolamine ($N = 12$, 1 mg/kg, s.c.) and 25 min later with milameline (1 mg/kg, s.c.). For this group, training for the passive avoidance test was performed 55 min after the administration of scopolamine.

Statistics

Pharmacological MRI

Statistical analyses were performed in SPM8. In a first level analysis pre- and post-injection conditions were compared for each subject. In a second level analysis, paired *T*-tests were used to compare pre-injection and post-injection conditions on a group level (uncorrected, $p < 0.001$).

Pharmacological resting-state functional MRI: ROI-based analysis

The statistical analyses for the correlation matrices were performed using MATLAB. One sample *T*-tests were performed for the mean correlation matrices of the saline and scopolamine groups to retain the significant correlations per group. The false discovery rate (FDR) correction was used to correct for multiple comparisons. Two sample *T*-tests were performed for the comparison of two groups to determine which functional correlations are significantly different ($p < 0.05$, uncorrected for multiple comparisons). For the saline vs. scopolamine group, a difference matrix was obtained showing the differences, represented as *T*-values, between both groups for all functional correlations. A color map of this matrix was produced in MATLAB, showing warmer colors for higher *T*-values i.e. for bigger differences between the groups.

Pharmacological resting-state functional MRI: seed-based correlation analysis

Mean statistical zFC-maps of each group were obtained in SPM8 using a one-sample *T*-test and were corrected for multiple comparisons using a FDR-correction ($p < 0.05$) and a threshold of at least 20 voxels. Comparison of the zFC-maps between groups was performed using a one-way ANOVA with a FDR-correction for multiple comparisons ($p < 0.05$) and a threshold of 20 voxels.

For the individual zFC-maps of the left hippocampus, cingulate cortex or visual cortex seed regions of each animal, a threshold was set to retain the significant functional connections with the seed region (FDR-correction for multiple comparisons, $p < 0.05$). The strength of FC between the seed region and other regions (cfr. Section 2.4.3) is represented as *T*-values for each subject and was analyzed in SPSS (http://www-01.ibm.com/software/be/analytics/spss/). To evaluate the dose-dependent effect of scopolamine, a one-way Anova was used with the Tukey correction for multiple comparisons ($p < 0.05$). To evaluate differences between the baseline, scopolamine and milameline conditions, a one-way Anova within-subject was implemented with the Tukey correction for multiple comparisons ($p < 0.05$).

Behavior study

The statistical analyses of the passive avoidance test were performed in SPSS using a paired *T*-test ($p < 0.05$) to evaluate differences between training and test day for each group.

Results

Pharmacological MRI

Muscarinic AChRs are present throughout the entire brain i.e. in cortical areas, the hippocampus, the thalamus etc. Pharmacological MRI (phMRI) was performed to identify brain regions where scopolamine induces T2* MRI signal intensity changes. Fig. 1 shows the phMRI results; pre-injection vs. post-injection statistical difference maps were obtained for the saline-injected (10 ml/kg) animals and the scopolamine-injected (3 mg/kg) animals. The doses were chosen based on the dose-range used in behavior studies described in the literature (Agrawal et al., 2009; Rush, 1988). For the saline-injected group there were no significant signal intensity differences between pre-injection and post-injection. For the scopolamine-injected group significant decreases in signal intensity were observed in several cortical regions, the hippocampus and the thalamus upon administration of scopolamine ($p = 0.001$). Based on these results, regions-of-interest (ROIs) were chosen for the pharmacological rsfMRI data analysis.

Pharmacological resting-state functional MRI: the effects of scopolamine on brain FC

Pharmacological rsfMRI was performed to evaluate the effects of scopolamine on FC: brain FC of saline-injected (10 ml/kg) and scopolamine-injected (3 mg/kg) groups was compared using a ROI-analysis. The ROIs were chosen based on the results of the phMRI and are shown in Fig. 1 i.e. the orbitofrontal cortex (OFC), the cingulate cortex (Cg), the somatosensory cortex (SS), the anterior thalamus (T), the hippocampus (HC), the retrosplenial cortex (Resp), the rhinal cortex (RC), the auditory cortex (AC) and the visual cortex (VC). The ventral tegmental area/substantia nigra (VTA/SN) was chosen as control region as it did not show significant signal intensity changes in the phMRI.

Functional connections between these ROIs are represented in correlation matrices (Fig. 2). Functional connections with the hippocampus, cingulate cortex and visual cortex were significantly affected by the administration of scopolamine: the hippocampus–thalamus ($p = 0.004$), the cingulate cortex–retrosplenial cortex ($p = 0.02$), the cingulate cortex–rhinal cortex ($p = 0.03$), the cingulate cortex–

visual cortex ($p = 0.01$), the visual cortex–retrosplenial cortex ($p = 0.02$) and the visual cortex–rhinal cortex ($p = 0.01$) functional connections.

Additionally a seed-based analysis was performed with the left hippocampus, cingulate cortex or visual cortex as seed regions (Fig. 3). This analysis resulted in mean zFC-maps for each group showing all significant functional connections with the seed region. For all seed regions the scopolamine-treated group showed overall significantly decreased FC compared to the saline-injected group (hippocampus, $p < 0.001$, FDR corrected; cingulate cortex, $p < 0.001$, FDR corrected; visual cortex, $p < 0.001$, FDR corrected). To account for peripheral effects of scopolamine on brain FC, a group of animals was injected with methyl-scopolamine (3 mg/kg), a scopolamine derivative with poor blood–brain–barrier permeability. The methyl-scopolamine-treated animals showed no significant differences compared to the saline-treated group (Fig. 3). Compared to the scopolamine-treated animals, significant increases of overall FC were observed for all seed-regions (hippocampus, $p < 0.01$, FDR corrected; cingulate cortex, $p < 0.05$, FDR corrected; visual cortex, $p < 0.01$, FDR corrected).

To evaluate whether the functional connections that were significantly decreased by scopolamine were affected in a dose-dependent manner, their FC strength was evaluated at 2 lower doses of scopolamine (0.5 mg/kg and 1 mg/kg). The measure for FC strength is a T-value, resulting from the individual FC-maps demonstrating all significant FC with the seed region i.e. the hippocampus, cingulate cortex or visual cortex (cfr. material and methods Section 2.4.3) per subject. The results (Fig. 4) confirmed a dose-dependent effect of scopolamine on FC, which reached statistical significance starting from the dose of 1 mg/kg for all the assessed functional connections. Furthermore, FC decreases reached statistical significance starting from the lowest dose of 0.5 mg/kg for FC between the cingulate and rhinal cortex, between the cingulate and visual cortex and between the visual and rhinal cortex.

Behavior and pharmacological rsfMRI: recovery of scopolamine-induced memory deficits and FC disruptions

Next, the passive avoidance behavior test was performed to evaluate whether scopolamine-induced memory deficits could be reversed with milameline. Passive avoidance behavior was assessed in the
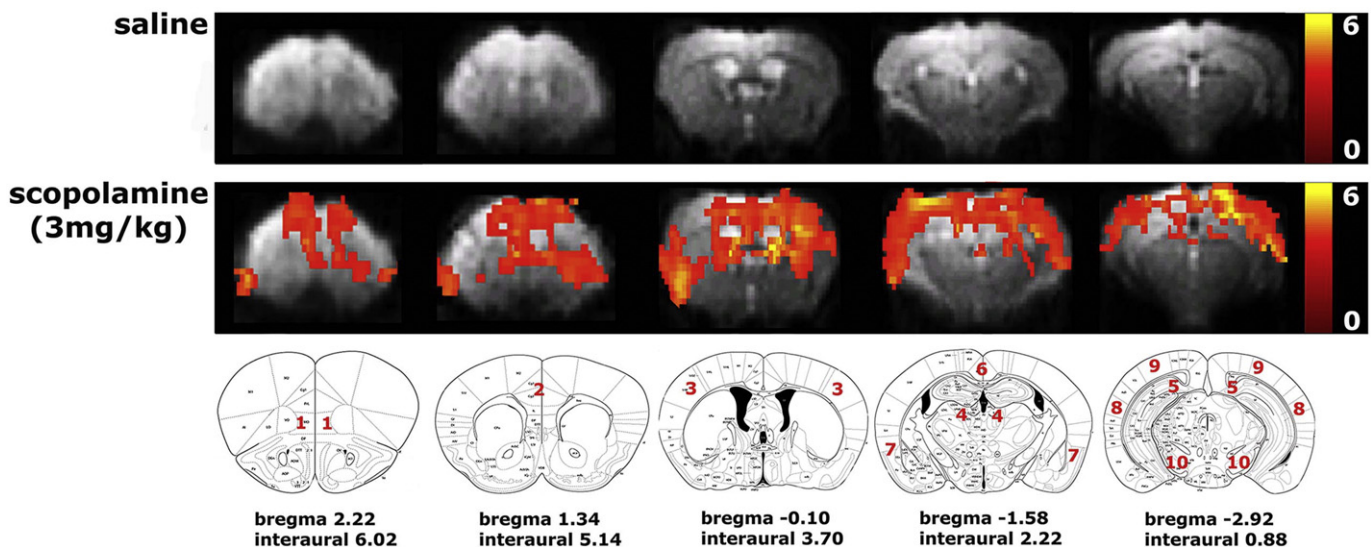

Fig. 1. PhMRI of the saline and scopolamine group. This figure shows five consecutive slices of the pre-injection vs. post-injection statistical difference maps for the saline and scopolamine injected group ($N = 10$/group). The color scale on the right indicates the T-value i.e. the strength of the difference between both conditions. The regions-of-interests chosen for the pharmacological rsfMRI analyses are indicated on the Franklin and Paxinos anatomical mouse brain atlas: 1 = orbitofrontal cortex, 2 = cingulate cortex, 3 = somatosensory cortex, 4 = anterior thalamus, 5 = hippocampus, 6 = retrosplenial cortex, 7 = rhinal cortex, 8 = auditory cortex, 9 = visual cortex, and 10 = ventral tegmental area/substantia nigra.
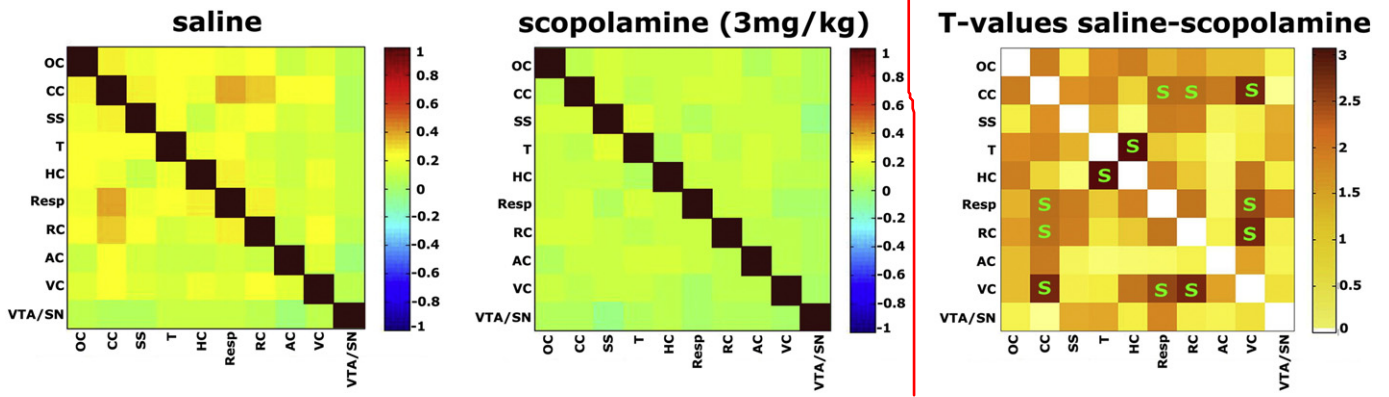
Fig. 2. The effect of scopolamine on brain FC. This figure shows the z-transformed FC matrices of the saline (left) and scopolamine (middle) group (N = 15/group). The color scale represents the strength of the functional connection. T-values representing the difference between the saline vs. scopolamine group are visualized in a color map (right), where higher T-values are represented by warmer colors. Abbreviations: OC = orbitofrontal cortex, CC = cingulate cortex, SS = somatosensory cortex, T = thalamus, HC = hippocampus, Resp = retrosplenial cortex, RC = rhinal cortex, AC = auditory cortex, VC = visual cortex, VTA/SN = ventral tegmental area/substantia nigra, and S = significant differences ($p < 0.05$).

following groups: saline (10 ml/kg), scopolamine (1 mg/kg), milameline (1 mg/kg) and in a group which was subsequently injected with scopolamine (1 mg/kg) and milameline (1 mg/kg). The dose of 1 mg/kg scopolamine was chosen because it induced significant FC differences for the six assessed functional connections. The dose of 1 mg/kg milameline was chosen based on the dose-range used in behavior studies described in the literature (Schwarz et al., 1999). The results (Fig. 5) showed a significant increase in latency for the saline (training day 11.2 ± 1.0 s; test day 208 ± 4 s; $p = 0.001$) and milameline (training day 24.8 ± 4.8 s; test day 166 ± 50 s; $p = 0.02$) injected groups. The scopolamine-injected group showed no significant change in latency (training day 51 ± 12 s; test day 62 ± 27 s). The group injected subsequently with scopolamine and milameline showed an increased latency (training day 32 ± 6 s; test day 103 ± 35 s) that reached a trend ($p = 0.065$).

Finally, pharmacological rsfMRI was performed using the same treatment protocol as the passive avoidance test to determine whether scopolamine-induced FC deficits could be reversed by milameline. Pharmacological rsfMRI scans were acquired subsequently at baseline, after the administration of scopolamine (1 mg/kg) and after the administration of milameline (1 mg/kg). For all the assessed functional connections scopolamine significantly decreased FC compared to baseline conditions (Fig. 5). FC between the hippocampus and thalamus, between the cingulate and retrosplenial cortex and between the visual and retrosplenial cortex was completely recovered after the subsequent administration of milameline. However, FC between the cingulate and rhinal cortex, between the cingulate and visual cortex and between the visual and rhinal cortex was not completely recovered by milameline and still showed significant differences with baseline (Fig. 5).

Discussion

The current study evaluated the effects of cholinergic modulation on FC in the mouse brain upon acute administration of the mAChR antagonist scopolamine and agonist milameline. This was done using pharmacological rsfMRI.

In the present study the hypothesis was that cholinergic modulations could be reflected as altered FC in the brain. This means that modulating the cholinergic system with scopolamine, which blocks cholinergic transmission, might induce FC disruptions in brain regions with a high abundance of mAChR. The phMRI study showed scopolamine-induced signal intensity changes in the cortex, the hippocampus and partly in the thalamus. It must be noted however that the signal changes in this part of the thalamus might possibly be due to spreading of the signal from the hippocampus. The statistical phMRI

map did not show the expected extensive signal intensity differences in all brain regions with a high density of cholinergic receptors. This could be explained by among others the use of anesthesia, which suppresses neuronal activation, or regional differences in baseline perfusion or neuronal activity due to differences in vascular density or reactivity. Nonetheless, these results are in line with studies demonstrating a high expression of mAChR proteins in the cortex and hippocampus using subtype-specific antibodies (Levey et al., 1991). The ventral tegmental area/substantia nigra region did not show signal intensity differences in the phMRI, probably due to the presence of distortions and the low signal-to-noise ratio. This region also did not show significant FC differences in the pharmacological rsfMRI analyses.

The pharmacological rsfMRI analyses revealed significant scopolamine-induced decreases of FC between the hippocampus and thalamus, between the cingulate and retrosplenial cortex, between the cingulate and rhinal cortex, between the cingulate and visual cortex, between the visual and retrosplenial cortex and between the visual and rhinal cortex. This is consistent with functional MRI studies in human subjects where altered hippocampal activity (Antonova et al., 2011; Sperling et al., 2002) and functional coherence (Wink et al., 2006) were observed upon scopolamine administration. Wink et al. showed increased FC between the hippocampus and frontal regions while the current study showed decreased FC. These discrepancies might be explained by differences in brain anatomy between human and rodents. Wink et al. focused mainly on FC between the hippocampus and frontal gyri, which cannot be identified in the brains of lissencephalic animals such as mice. Furthermore, there are metabolic differences between humans and rodents that might result in different effects of drugs administered in a similar dose and via the same route of administration. Despite these differences, Wink et al. still show that altering cholinergic neurotransmission affects FC in the brain. Although rsfMRI studies targeting the cholinergic system in rodents are very limited, there are fMRI studies in rats showing altered brain activity in frontal regions after the administration of scopolamine (Kocsis et al., 2014). The cholinergic network is known to be involved in learning and memory and the functional connections that are affected by scopolamine are involved in higher cognitive functions such as spatial memory, episodic memory, contextual memory, attention etc. (Aggleton et al., 2010; Burwell et al., 2004; Fell et al., 2006a,b; Fernandez and Tendolkar, 2006; Law and Smith, 2012; Leech and Sharp, 2014; Lenartowicz and McIntosh, 2005; Marchand et al., 2013; Teixeira et al., 2006). Countless behavior studies in humans and rodents (Hasselmo, 2006; Klinkenberg and Blokland, 2010) show that blockade of cholinergic synaptic transmission leads to disruption of these types of memory functions (Klinkenberg and Blokland, 2010). The results of the
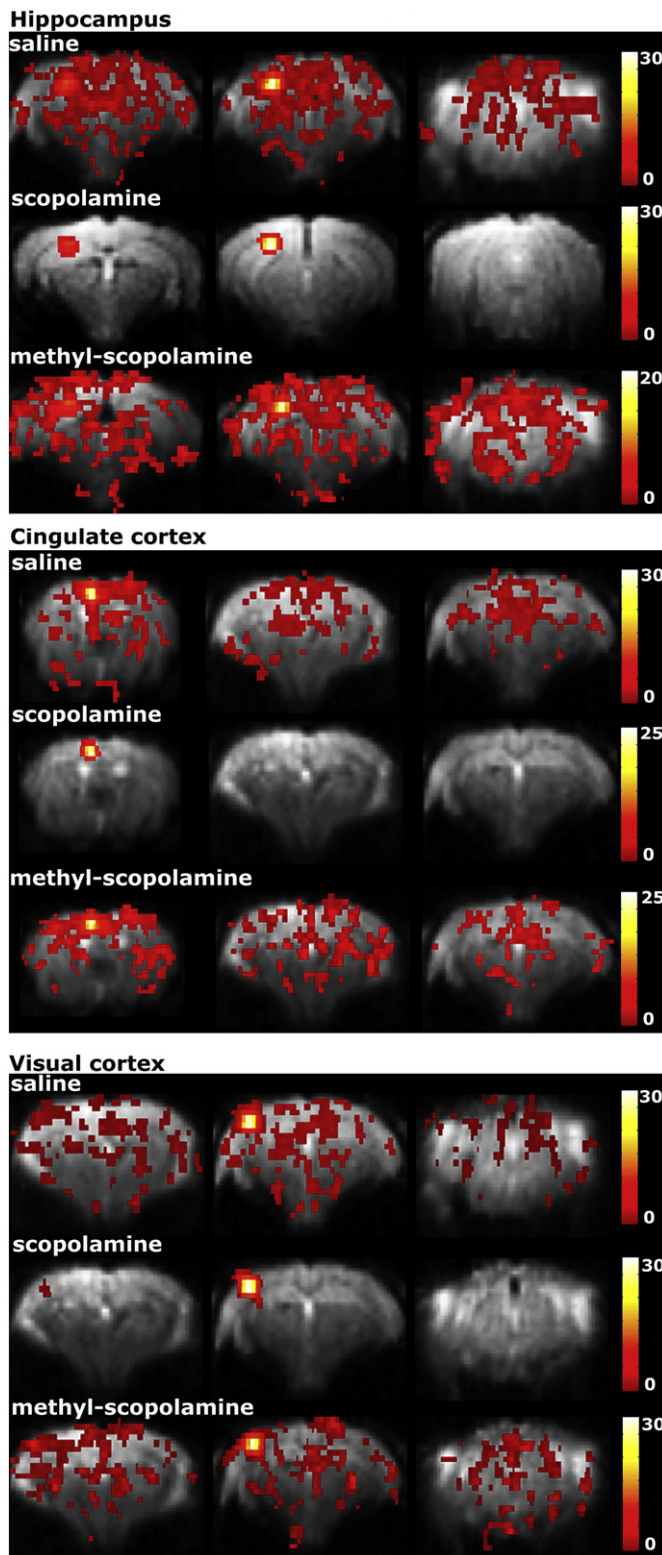
Hippocampus

Cingulate cortex

Visual cortex

Fig. 3. Seed-based analyses of the hippocampus, cingulate cortex and visual cortex. This figure shows three consecutive slices from the mean zFC-maps of the saline, scopolamine and methyl-scopolamine groups ($N = 15$/group), showing all significant functional connections with the seed i.e. the left hippocampus, cingulate cortex or visual cortex ($p < 0.05$, FDR corrected). The color scale represents the *T*-value i.e. the strength of the functional connection.

passive avoidance test performed in the current study also established that blocking the cholinergic system with scopolamine induces impairments of contextual memory.
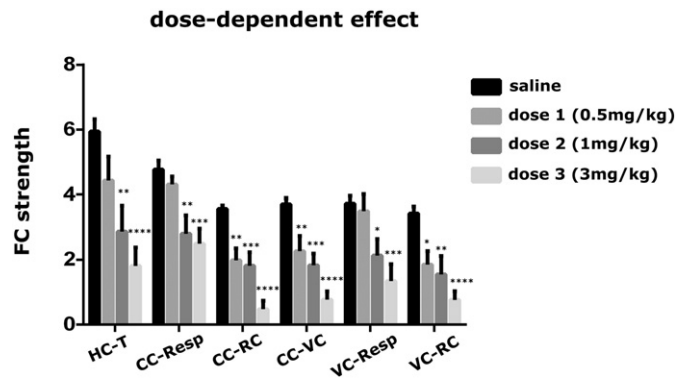

Fig. 4. Dose-dependent effect of scopolamine on FC. The graphs show the strength of FC ± standard error for the saline (10 ml/kg) and scopolamine-injected (0.5; 1; 3 mg/kg) group ($N = 15$/group) for the functional connection between the hippocampus (HC) and thalamus (T), the cingulate cortex (CC) and retrosplenial cortex (Resp), the cingulate cortex and rhinal cortex (RC), the cingulate cortex and visual cortex (VC), the visual cortex and retrosplenial cortex and between the visual cortex and rhinal cortex. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

The six functional connections that were significantly affected by scopolamine at 3 mg/kg were additionally evaluated at two lower doses of scopolamine. The results showed a dose-dependent response of scopolamine on FC which reached statistical significance for all functional connections at 1 mg/kg. Additionally, FC between the cingulate and rhinal cortex, between the cingulate and visual cortex and between the visual and rhinal cortex was significantly decreased by the lowest dose of 0.5 mg/kg. This could mean that those specific functional connections are particularly vulnerable to cholinergic blockade. This is possibly due to a higher mAChR density in those brain regions. Several studies confirm a high mAChR density in cortical regions of the rodent brain (Frey and Howland, 1992; Lein et al., 2007; Mulholland et al., 1992) but rigorous quantitative analyses of these specific brain regions would be necessary to correlate receptor density to the FC results of this study. Nonetheless, these results show that the effect of cholinergic blockade on FC can already be observed at lower doses in those regions, which has implications for early-stage studies in neurodegenerative diseases. Moreover, these results are consistent with findings in AD, where the cholinergic system is severely affected (Bales et al., 2006; Pepeu and Giovannini, 2004; Schneider et al., 2014; Watanabe et al., 2009). For example the cingulate cortex, which is a memory region, consistently shows altered FC (Sheline and Raichle, 2013) in AD patients. Furthermore, the rhinal cortex and visual cortex are involved in visual recognition and declarative memory, which is preferentially affected at an early stage during AD progression (Blaizot et al., 2002).

Next it was evaluated whether scopolamine-induced FC deficits could be reversed with milameline, which stimulates the cholinergic system. Milameline has been used in behavior studies where it improved scopolamine- or lesion-induced behavior deficits in rhesus monkeys and in rodents, (Heidrich et al., 1997; Schwarz et al., 1999). In the present study this was confirmed in mice using the passive avoidance test, which investigates contextual memory. The passive avoidance test showed that scopolamine-induced disruptions of contextual memory can be partially recuperated with milameline. A clear increase in latency was observed on the test day which reached a trend, but not statistical significance. The pharmacological rsfMRI analyses showed that milameline caused an increase in brain FC (Fig. S1), explaining how it might be able to recover FC decreases induced by scopolamine. It was established that the effects of scopolamine on brain FC persist for at least until 1 h after the injection, excluding that expected increase of FC after milameline treatment is caused by wash-out of scopolamine (Fig. S2). The pharmacological rsfMRI showed that scopolamine-induced FC deficits could be completely recovered for FC between the hippocampus and thalamus, between the cingulate and retrosplenial cortex and between the visual and retrosplenial cortex. However, for
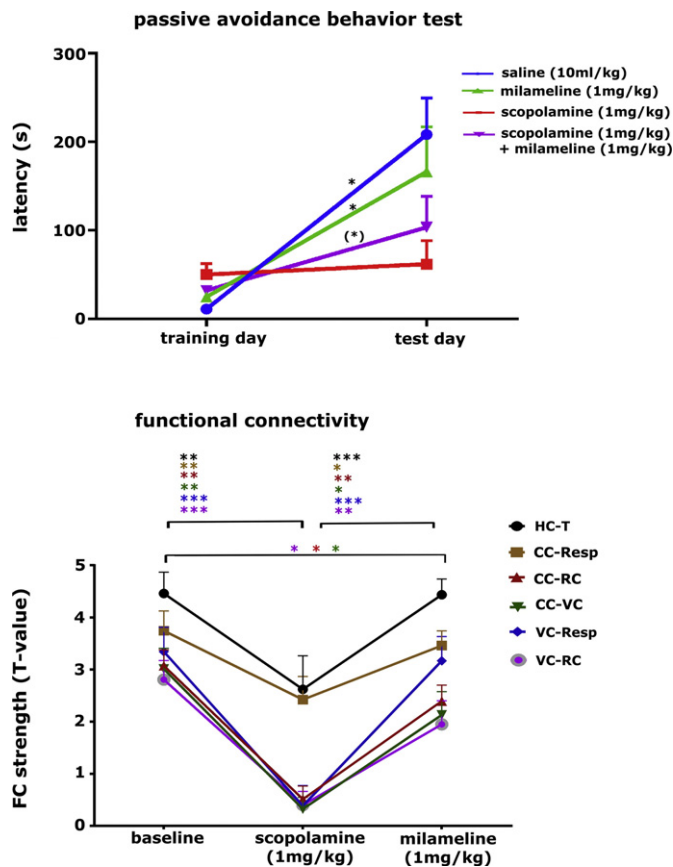
Fig. 5. Scopolamine-induced behavior and FC deficits can be reversed by milameline. Upper panel shows the results of the passive avoidance test for the saline (10 ml/kg), scopolamine (1 mg/kg), milameline (1 mg/kg) treated groups ($N = 8$/group) and for a group treated subsequently with scopolamine and milameline ($N = 12$). ** $p < 0.05$ and * $p < 0.1$. Lower panel shows a graph displaying the strength of the FC at baseline, after the injection of scopolamine and after the subsequent injection of milameline. FC strength ± standard error are shown for FC between the hippocampus (HC) and thalamus (T), the cingulate cortex (CC) and retrosplenial cortex (Resp), the cingulate cortex and rhinal cortex (RC), the cingulate cortex and visual cortex (VC), the visual cortex and retrosplenial cortex and between the visual cortex and rhinal cortex. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

FC between the cingulate and rhinal cortex, between the cingulate and visual cortex and between the visual and rhinal cortex milameline only partially recovered the scopolamine-induced FC deficits. This is consistent with the results of the behavior study, where milameline only partially recovered scopolamine-induced behavior deficits. Contextual memory depends on several brain regions including the hippocampus (Fanselow, 2000), thalamus (Marchand et al., 2013), cingulate cortex (Riekkinen et al., 1995), rhinal cortex (Burwell et al., 2004) and visual cortex (Aquino-Cias et al., 1972). Partial recovery of passive avoidance behavior by milameline can be explained by the fact that some functional connections involved in contextual memory show full recovery, but others only show partial recovery. These results demonstrate that pharmacological rsfMRI can detect the recovery of cholinergic blockade by stimulating the cholinergic system. This is consistent with studies in humans, where cholinesterase inhibitors, which also stimulate the cholinergic system, improved FC in the cingulate cortex (Li et al., 2012) and hippocampus (Pa et al., 2013; Risacher et al., 2013).

An interesting observation is that the functional connections that already showed decreased FC after administration of the lowest dose of scopolamine (cingulate–rhinal cortex, cingulate–visual cortex, visual–rhinal cortex) could also not be recovered completely by milameline. These observations further support the idea that those functional connections are particularly vulnerable. Furthermore, these functional

connections might not be easily recovered by treatment, suggesting that efforts should be made to develop treatment regimes that can efficiently target the cholinergic system in these specific brain regions.

It has to be taken into account that scopolamine has peripheral effects on cardiovascular function and causes vasoconstriction (Klinkenberg and Blokland, 2010). This means that the observed effects of scopolamine on brain FC are possibly a combination of peripheral and central effects. To evaluate the peripheral effects of scopolamine on brain FC, a group of mice was administered methyl-scopolamine. This is a methylated derivative of scopolamine which shows the same receptor binding characteristics as scopolamine but does not readily cross the blood–brain–barrier (Klinkenberg and Blokland, 2010). Several behavior studies that use scopolamine in rodents add an experimental group that is treated with an equivalent dose of methyl-scopolamine (Harvey et al., 1983; Pradhan and Roth, 1968). So if scopolamine induces behavior changes at a given dose and methyl-scopolamine does not affect behavior at the same dose, it can be assumed that the effects induced by scopolamine are mediated centrally and not peripherally. The current study showed that scopolamine induced FC deficits in the mouse brain at a given dose and methyl-scopolamine did not induce FC alterations at the same dose. This allows us to assume that the peripheral effects of scopolamine on cardiovascular function and vasoconstriction can be excluded, and that the observed effects are of a central nature. In contrast with the results of this study, recent work showed that butyl-scopolamine, which is also a derivative of scopolamine with poor blood–brain–barrier permeability, induced similar fMRI brain activation changes in the rat brain as scopolamine (Kocsis et al., 2014). That study concluded that the effects of scopolamine are probably mainly due to vascular responses. Another study, however, showed that butyl-scopolamine did not induce brain activation changes measured with fMRI (Cash et al., 2005). Differences between these studies include anesthesia protocols i.e. isoflurane vs. alfa-chloralose, route of administration i.e. intravenous vs. intraperitoneally and dosage. The current study showed that under medetomidine sedation methyl-scopolamine did not induce FC deficits in the mouse brain when administered subcutaneously at a dose of 3 mg/kg, while the same dose of scopolamine induced marked FC decreases. Therefore, we can conclude that the effects induced by scopolamine on mouse brain FC in this study are probably not dominated by peripheral effects.

On one hand, cholinergic modulators can influence neuronal activity at the level of the synapses (Wess, 2004, 2012). This has been demonstrated by electrophysiological studies showing slowing of neuronal activity by the administration of scopolamine (Ebert and Kirch, 1998; Kikuchi et al., 1999; Snaedal et al., 2010). Electrophysiological studies in mice (Brazhnik et al., 2003, 2004) showed that scopolamine decreases neuronal firing in the hippocampus. On the other hand, cholinergic modulators can affect the vasculature directly (Sato and Sato, 1995). Acetylcholine or cholinergic agonists cause vasodilatation and cholinergic antagonists cause vasoconstriction. There are five types of mAChR i.e. M1–M5 and several studies have tried to unravel the specific location and function of each of these receptors in the brain (Levey et al., 1991; Tice et al., 1996). Scopolamine is a non-selective mAChR antagonist which means that it binds to all mAChR subtypes with comparable affinity (Golding and Stott, 1997). Studies in a M5-receptor knock-out mouse model demonstrated that the administration of ACh in these mice did not induce any vasodilatation in the cerebral vasculature, suggesting that the M5-receptor subtype is responsible for vasoactive functions of ACh in the brain (Yamada et al., 2001, 2003). So scopolamine will probably induce cerebral vasoconstriction through the M5 receptor subtype, but synaptic activity will be modulated through all of the other mAChR subtypes which are abundantly present throughout the whole brain (Lein et al., 2007). Consistent with this notion, in AD, soluble amyloid exerts toxic effects at the level of among others the cholinergic neurons. These toxic effects influence synaptic transmission of the cholinergic system in AD pathology (Auld et al., 2002; Buttini et al., 2002; Mesulam, 2004; Ohno et al.,

2004; Yan and Feng, 2004). Furthermore, the eventual loss of cholinergic synaptic function will play a considerable role in the CBF decreases observed in AD patients and mouse models of AD (Kuznetsova and Schliebs, 2013; Ongali et al., 2010; van Beek and Claassen, 2011). The BOLD signal depends on among others cerebral blood flow (CBF), which in turn can be influenced by changes in neuronal activity at the level of the synapses or by a direct vasoactive at the level of the brain vessels. Regarding the cholinergic system, it is not straightforward to completely separate these two effects.

In conclusion, the present study demonstrates that blocking the cholinergic system is reflected as decreased FC in the mouse brain that can be detected dose-dependently using pharmacological rsfMRI. Moreover, stimulating the cholinergic system after blockade can recover the functional connections that were affected. Some functional connections could be recovered completely and others only partially, which correlated with the partial recovery of contextual memory deficits by cholinergic stimulation. Finally, this study revealed that some functional connections in the cholinergic network are especially vulnerable to modulation, which has implications for read-out and treatment strategies in diseases where the cholinergic system is affected, like in mouse models of AD. It has to be mentioned that the effect of acute pharmacological modulations are not necessarily comparable to the effect of chronic modulations as in pathological conditions. Future studies could focus on the effect of chronic modulations of the cholinergic system and assess whether they can be reversed by treatment. Nonetheless, the results of this study have implications for studies in mouse models of neurodegenerative disorders, where pharmacological rsfMRI might possibly be used as a non-invasive tool to detect early alterations of neurotransmitter systems induced by disease or treatment.

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FIG. S1
The effect of scopolamine and milameline on brain FC. This figure shows the z-transformed FC matrices of the saline (left), scopolamine (middle) and milameline (right) group (N = 15/group). The color scale represents the strength of the functional connection. Abbreviations: OC = orbitofrontal cortex, CC = cingulate cortex, SS = somatosensory cortex, T = thalamus, HC = hippocampus, Resp = retrosplenial cortex, RC = rhinal cortex, AC = auditory cortex, VC = visual cortex, and VTA/SN = ventral tegmental area/substantia nigra.

FIG. S2
Effect of scopolamine (1 mg/kg) on FC at 25 min and 60 min post-injection. The graphs show the strength of FC for 2 groups injected with scopolamine (1 mg/kg) at 25 min and 60 min post-injection. FC strength ± standard error are shown for FC between the hippocampus (HC) and thalamus (T), the cingulate cortex (CC) and retrosplenial cortex (Resp), the cingulate cortex and rhinal cortex (RC), the cingulate cortex and visual cortex (VC), the visual cortex and retrosplenial cortex and between the visual cortex and rhinal cortex.