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Modulation of nucleus accumbens connectivity by alcohol drinking and naltrexone in alcohol-preferring rats: A manganese-enhanced magnetic resonance imaging study

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Abstract

The nonselective opioid receptor antagonist naltrexone is now used for the treatment of alcoholism, yet naltrexone's central mechanism of action remains poorly understood. One line of evidence suggests that opioid antagonists regulate alcohol drinking through interaction with the mesolimbic dopamine system. Hence, our goal here was to examine the role of the nucleus accumbens connectivity in alcohol reinforcement and naltrexone's actions using manganese-enhanced magnetic resonance imaging (MEMRI). Following long-term free-choice drinking of alcohol and water, AA (Alko Alcohol) rats received injections of $MnCl_2$ into the nucleus accumbens for activity-dependent tracing of accumbal connections. Immediately after the accumbal injections, rats were imaged using MEMRI, and then allowed to drink either alcohol or water for the next 24 h. Naltrexone was administered prior to the active dark period, and the second MEMRI was performed 24 h after the first scan. Comparison of signal intensity at 1 and 24 h after accumbal $MnCl_2$ injections revealed an ipsilateral continuum through the ventral pallidum, bed nucleus of the stria terminalis, globus pallidus, and lateral hypothalamus to the substantia nigra and ventral tegmental area. Activation was also seen in the rostral part of the insular cortex and regions of the prefrontal cortex. Alcohol drinking resulted in enhanced activation of these connections, whereas naltrexone suppressed alcohol-induced activity. These

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data support the involvement of the accumbal connections in alcohol reinforcement and mediation of naltrexone's suppressive effects on alcohol drinking through their deactivation.
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1. Introduction

The endogenous opioid system is implicated in the reinforcing and psychomotor stimulant properties of alcohol (Herz, 1997). Since the nonselective opioid antagonist naltrexone was first demonstrated to attenuate alcohol reinforcement (Altshuler et al., 1980), opioid antagonists have been shown to suppress alcohol self-administration, alcohol seeking and relapse in various experimental models (Hyytiä and Kiiänmaa, 2001; Katner et al., 1999; Kornet et al., 1991). In human alcoholics, opioid antagonism with naltrexone and nalmeferine reduce alcohol consumption, craving, and relapse (Mann et al., 2013; Volpicelli et al., 1992).

Although the neurobiological mechanisms underlying the regulation of alcohol-related behaviors by opioid antagonists are not completely understood, there is evidence to suggest that the effects of opioid antagonists could be mediated partly through an interaction with the mesolimbic dopamine system. In rodents, alcohol administration causes the release of β -endorphin in the key areas of the mesolimbic dopamine system, including the ventral tegmental area (VTA) and nucleus accumbens (Jarjour et al., 2009; Marinelli et al., 2003). Since the VTA μ -opioid receptor activation by endogenous opioid peptides is hypothesized to suppress the inhibitory GABAergic interneurons (Johnson and North, 1992; Spanagel et al., 1992), alcohol-induced release of opioid peptides could contribute to the disinhibition of VTA dopamine neurons and increase dopamine release in the nucleus accumbens (Di Chiara and Imperato, 1988). Consistent with this hypothesis, opioid antagonists administered either systemically or locally into the nucleus accumbens or VTA decrease alcohol-induced accumbal dopamine release (Benjamin et al., 1993; Gonzales and Weiss, 1998; Valenta et al., 2013).

In line with the preclinical findings, naltrexone has been demonstrated to diminish cue-induced activation of the ventral striatum in humans (Myrick et al., 2008), highlighting the potential for neuroimaging as a tool for clarifying the neurobiological mechanisms underlying the effects of therapeutic drugs and for finding neural correlates of positive treatment effects (Konova et al., 2013; Schacht et al., 2013a). Alcohol-related neuronal activation patterns could be employed as translational biomarkers for bridging preclinical animal and clinical research, because they supposedly closely reflect neurobiological mechanisms underlying alcohol effects and their modulation by medications. Despite the general limitations for functional magnetic resonance imaging (MRI) in rodents, manganese-enhanced MRI (MEMRI) offers a feasible approach to functional imaging. In MEMRI, accumulation of paramagnetic Mn^{2+} ions into excitable cells during depolarization through

voltage-gated calcium channels and the slow Mn^{2+} efflux allows functional brain imaging in anesthetized animals that have previously undergone behavioral or pharmacological testing (Dudek et al., 2014; Eschenko et al., 2010; Lin and Koretsky, 1997). In addition, Mn^{2+} ions are transported along the axons, released from the terminals into the synaptic cleft, and taken up by the postsynaptic neuron, allowing tracing of monosynaptic or even multisynaptic neuronal connections (Canals et al., 2008; Inoue et al., 2011; Pautler and Koretsky, 2002).

We have previously demonstrated with MEMRI that voluntary alcohol drinking induces widespread functional brain activation in the alcohol-preferring AA (Alko Alcohol) rats (Dudek et al., 2014). This rat line is one of the best described animal models of high alcohol drinking, achieving pharmacologically relevant blood alcohol levels during drinking sessions (Sommer et al., 2006). Prompted by previous findings on the potential role of the mesolimbic dopamine system in alcohol reward and the actions of opioid antagonists, we aimed here at characterizing alcohol-induced activation and the pharmacological modulation thereof by naltrexone in the projections of the nucleus accumbens. Combining both properties of Mn^{2+} ions, namely their activity-dependent uptake and anterograde transport in axons, we hypothesized that after being injected into the nucleus accumbens, accumulation of Mn^{2+} uptake and accumulation seen as enhanced signal intensity in accumbal connections will reveal the neuroanatomical substrate for the actions of alcohol and opioid antagonists.

2. Experimental procedures

2.1. Animals

Male alcohol-preferring AA (Alko, Alcohol) rats, weighing 200–300 g at the beginning of the experiments were used. All rats were 6 weeks old at arrival and were single-caged in transparent, individually ventilated cages lined with sawdust bedding. The cages contained a wooden stick and nesting material as enrichment. Food (SDS, Witham, UK) and tap water were freely available in the home cages at all times, except for the first four days of alcohol drinking (see below). Animals were housed in a room with a controlled temperature $21 \pm 1^\circ\text{C}$ and relative humidity $55 \pm 10\%$ on a 12-h light/dark cycle with lights on at 6:00. All experiments were performed during the light phase of the cycle. All experimental procedures using animals were conducted in accordance with directive 2010/63/EU of the European Parliament and of the Council and the Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013) and were approved by the project authorization board of the Regional State Administrative Agency for Southern Finland.

2.2. Voluntary alcohol consumption

Drinking fluids were provided in 250 ml drinking bottles equipped with stainless steel spouts, placed on cage covers. During the first four days, rats were given 10% (v/v) ethanol solution as their only drinking fluid to habituate them to the taste of alcohol. For the next six weeks, all rats were allowed a two-bottle choice between 10% alcohol and water. Once a week, consumption of the fluids and the body weights were recorded and the bottles were filled with fresh solutions. The water-drinking experimental group had always two water bottles available, and their weekly maintenance was identical to that of the alcohol-drinking rats.

2.3. Intra-accumbal MnCl₂ administration

Rats were anesthetized with 5% isoflurane and placed in a stereotaxic instrument with the incisor bar positioned at 3.3 mm below the interaural line. Anesthesia was then maintained with 2% isoflurane during the intracerebral injection and subsequent MRI scanning. Injections of MnCl₂ were aimed at the nucleus accumbens using the following stereotaxic coordinates chosen with reference to the atlas of Paxinos and Watson (2007): +1.9 from the bregma, -1.5 from the midline, -7.2 from the skull surface. The selected MnCl₂ concentration of 100 mM and the injection volume of 50 nl were identical or lower than the previously determined thresholds under which no neuronal cell death or astrogliosis could be seen (Canals et al., 2008). The injections were performed with a 0.5 µl Hamilton syringe with a 26 G needle over a period of 40 min, resulting in an infusion rate of 1.25 nl/min. After completing the injection, the needle was left in the brain for 10 min, whereupon it was slowly retracted over a period of 2 min. The drill hole in the skull was closed with bone wax and the scalp was sutured. The acquisition of the baseline MEMRI scans was initiated immediately following the intracerebral MnCl₂ injections.

2.4. Experimental design

The experimental design and groups are depicted in Figure 1. The following experimental groups were studied: (1) the group drinking water continually ($N=9$), (2) the group with a history of continual two-bottle choice alcohol drinking withdrawn from alcohol for seven days ($N=9$), (3) the group drinking 10% alcohol continually in a two-bottle choice situation, injected acutely with saline ($N=9$), (4) the alcohol-drinking group, injected acutely with a 3 mg/kg dose of naltrexone ($N=10$). There were no statistically significant differences in the basal alcohol consumption between the alcohol-drinking groups before MRI scanning (abstinent group: 5.65 ± 0.39 g/kg/day; saline-treated group: 6.43 ± 0.36 g/kg/day; naltrexone-treated group: 6.52 ± 0.59 g/kg/day; $F_{2,28}=0.99$, $P=0.38$). Intra-accumbal MnCl₂ injections were given during the light phase of the 12-h light/dark cycle. Immediately after termination of injections, all rats were imaged as described (see above), followed by a second imaging session 24 ± 1 h later at the same time. Free access to water or alcohol was never limited, except during surgery and imaging. For evaluating the effects of naltrexone on the activity of accumbal connections, alcohol-drinking rats were injected either with saline (Group 3) or naltrexone (Group 4) 30 min before the onset of the dark phase, during which rats had free choice to water and alcohol (see Figure 1B). It has previously been shown that AA rats consume most of their alcohol during their active dark phase (Hyytiä and Sinclair, 1990). The injections were given 3–5 h after the baseline scan, with the interval length counterbalanced between the experimental groups.

Figure 1 The design of the experiments. Panel A presents the experimental groups included in the study. For detailed description of the groups and procedures, see Sections 2 and 2.4. The panel B is a depiction of the final 24-h period for the experimental groups 3 and 4, showing injections of saline or naltrexone immediately before the 12-h dark phase during which the rats had free choice to water and alcohol.

2.5. MRI data acquisition

During the scanning procedure rats were anesthetized with 5% isoflurane in oxygen (0.8–1 L/min) and placed on a custom-made holder with a tooth bar and a nose cone for stabilization. After reaching a desired level of anesthesia isoflurane concentration was reduced to 2%–3% and maintained at this level for the remainder of scanning. Body temperature was kept constant with a heating pad. Magnetic resonance imaging was performed on a 4.7 T scanner (Bruker, PharmaScan 47/16 US, Ettlingen, Germany) using a 38 mm linear volume coil for transmit and receive. T1-weighted images were acquired using a three-dimensional rapid acquisition-relaxation enhanced (RARE) pulse sequence (repetition time=300 ms, effective echo time=12.5 ms, number of averages=7, number of slices: coronal=127, sagittal=57, axial=57, flip angle=180°, field of view $26 \times 17 \times 17$ mm³, and matrix size=128/54/54, resulting in $0.2 \times 0.31 \times 0.31$ mm³ voxel resolution).

2.6. Data analysis

All MRI images were converted to Analyze format, scaled up by a factor of 10 and spatially pre-processed with a custom-developed MATLAB functions (version R2014a). Briefly, T1-weighted and brain-extracted images were spatially normalized using a rat brain template co-registered to a rat brain atlas (Schwarz et al., 2006) by a 12-parameter affine transformation using the FSL/ FLIRT tool (Jenkinson et al., 2002). This template was co-registered to the digitized Paxinos and Watson atlas (4th ed., 1998), enabling atlas-based generation of region-of-interest (ROI) masks for detailed anatomical analysis.

For mapping the distribution of significant voxels in experimental groups receiving intra-accumbal MnCl₂ injections, statistical parametric maps were generated by implementing voxel-wise paired *t*-tests comparing the 1-h baseline scans with scans acquired 24 h later using SPM8 (www.fil.ion.ucl.ac.uk/spm). In order to estimate the amount of MnCl₂ delivered by injections, the T1-weighted signal intensity in the ROI representing the nucleus accumbens was extracted from the individual 1-h baseline scans. One-way ANOVA

detected no significant differences in the mean accumbal signal intensity between the experimental groups ($F_{3,37}=0.07$, $P=0.98$). In addition, no differences were seen either in the mean signal intensity of the whole injection plume defined with an identical intensity threshold in all groups ($F_{3,37}=0.47$, $P=0.71$) or in the mean number of voxels defining the injection plume ($F_{3,37}=0.42$, $P=0.74$). For creating statistical parametric maps by SPM, the image intensity in each individual scan was then normalized using the nucleus accumbens signal intensity in order to correct small intra-individual differences in the amount of injected MnCl_2 (Canals et al., 2008; Daoust et al., 2014). The significance threshold for individual voxels was $P<0.0001$ (uncorrected). For characterizing further the anatomical distribution of Mn^{2+} labeled voxels, the percentage of the voxels displaying statistically significant signal intensity increase ($P<0.0001$) was calculated in predefined ROIs extracted by the REX tool (Duff et al., 2007). For analyzing activation differences between the experimental groups, a voxel wise ROI analysis was performed by conducting preplanned (water vs. abstinence; water vs. alcohol, alcohol vs. naltrexone, water vs. naltrexone) 2-tailed t -tests between the groups by SPM and applying three-dimensional anatomical masks for data extraction by the REX tool.

2. Results

The MRI scans acquired immediately after the intra-accumbal MnCl_2 injections allowed anatomical verification of injector locations. As shown in Figure 2A, the injection needle tips were located both in the core and shell regions of the nucleus accumbens, and therefore the pattern of Mn^{2+} intake and transport probably reflects the activity of neurons in both accumbal regions. Furthermore, there were no systematic differences between the groups in the distribution of injections along the rostral-caudal dimension. In the typical subjects (Figure 2B), the injection plumes seen 1 h after the termination of the injection indicated that although most injections were mostly confined within the anatomical boundaries of the nucleus accumbens, partial diffusion was also seen dorsal to accumbens, i.e., in the ventromedial caudate-putamen. Figure 2C shows that there were no differences in the T1-weighted signal intensity in the ROI representing the nucleus accumbens between the experimental groups ($F_{3,37}=0.07$, $P=0.98$).

Figure 3 shows the statistical parametric maps displaying statistically significant ($P<0.0001$) differences between T1-signal intensities recorded at 1 and 24 h time points following intra-accumbal MnCl_2 injections. Rats engaged in drinking water, alcohol, and abstaining from continual alcohol drinking exhibited an anatomically confined rostral-caudal activation. Given the estimated axonal transport velocity of Mn^{2+} ions, 2.8 mm/h (Watanabe et al., 2004), the 24-h imaging interval allowed mapping of all mono-synaptic connections. Highest activation was observed in areas directly adjacent to the nucleus accumbens, such as ventromedial caudate-putamen, ventral pallidum, and bed nucleus of the stria terminalis. Caudally, this cluster included the medial forebrain bundle, i.e., mostly the lateral hypothalamus and zona incerta, extending to the substantia nigra and lateral parts of the ventral tegmental area. In rats allowed to drink alcohol during the 24-h period between the imaging sessions, activation of the medial forebrain bundle was enhanced, and additional areas, such as the insular cortex, somatosensory cortex, amygdala, and pedunclopontine tegmental nucleus were activated. These within-group data were also supported by between-group analysis that showed significantly higher activation in the alcohol- than the water-drinking rats in the insula, somatosensory and motor cortex, amygdala, zona incerta, and pedunclopontine tegmental nucleus (Table 1). Also the slightly lower activation in the abstinent group shown in Figure 3 was confirmed by the ROI analyses, revealing significantly lower activation the orbitofrontal cortex, prefrontal cortex, and insula in the alcohol abstinent rats compared to the water-drinking animals (Table 1). On the other hand, early abstinence was accompanied by increased activity in the amygdala.

In a sharp contrast to the aforementioned experimental groups, alcohol-drinking rats given an acute naltrexone injection (3 mg/kg, s.c.) displayed a remarkably diminished pattern of activation seen both in the horizontal and coronal sections. Table 1 shows the comparison between saline- and naltrexone-treated alcohol-drinking rats and indicates significantly decreased activity by naltrexone in the orbital and insular cortex, caudate-putamen, and amygdala. A trend towards suppressed activity was seen in the

Figure 2 (A) Locations of injector tips determined from T1-weighted images acquired immediately after the MnCl_2 injections in subjects belonging to the four experimental groups. Numbers beside the sections indicate the positions of the sections from bregma in millimeters (Paxinos and Watson, 2007). (B) Representative T1-weighted MRI images of injection plumes immediately after injections at two different levels from the bregma. (C) The mean ($\pm$ SEM) T1 signal intensity at the nucleus accumbens ROI in the experimental groups determined from the T1 scans after intra-accumbal MnCl_2 injections.

Figure 3 Increased intensity of T1-weighted MRI signal measured 24 hrs after MnCl_2 injections into the nucleus accumbens. Statistical maps ($P < 0.0001$, uncorrected) from paired t -tests ($N = 9-10/\text{group}$) comparing the 1-h images with 24-h images within each individual are superimposed on T2-weighted horizontal and coronal sections from the brain template (Schwarz et al., 2006), with the corresponding atlas sections manually overlaid. Note that for easier viewing, the scale of the horizontal sections is slightly larger than that of the coronal sections. Numbers above the coronal sections indicate the positions of the sections from bregma in millimeters. Abbreviations used for brain regions: VP, ventral pallidum; CPu, caudate putamen; LH, lateral hypothalamus; INS, insular cortex; SN, substantia nigra; O, orbital cortex; PrL, prelimbic cortex; BST, bed nucleus of stria terminalis; ZI, zona incerta; Amy, amygdala; S2, secondary somatosensory cortex; Ent, entorhinal cortex; PPTg, pedunculo-pontine tegmental nucleus.

somatosensory and motor cortex, globus pallidus, ventral pallidum, and zona incerta. Compared with the water-drinking rats, naltrexone-treated animals exhibited no deactivation, whereas naltrexone activated the somatosensory cortex and pedunculo-pontine nucleus.

As alcohol drinking was measured following the naltrexone injection, we attempted to evaluate the relationship between naltrexone-induced changes in functional activity and alcohol drinking. However, this analysis was confounded by the anesthesia and surgical procedures during the MnCl_2 injection and the baseline scanning. Figure 4A shows that the saline-treated rats decreased their alcohol intake by

$22 \pm 6\%$ ($t_8 = 3.16$, $P = 0.013$), whereas alcohol intake decreased by $40 \pm 6\%$ ($t_9 = 5.77$, $P = 0.0003$) in the naltrexone-treated group compared to the pre-treatment baseline. Suppression of alcohol drinking by naltrexone was confirmed by a significant interaction term (day $\times$ treatment, $F_{1,17} = 5.96$, $P = 0.041$) by a two-way ANOVA with day as the within-subjects and treatment as the between-subjects factor. During the 24-h interval, there was a compensatory increase in water drinking (Figure 4B), revealed by a significant main effect of day ($F_{1,17} = 5.13$, $P = 0.037$), but no differences between the saline- and naltrexone-treated rats (day $\times$ treatment, $F_{1,17} = 1.11$,

Table 1 *T*- and *p*-values from preplanned voxelwise *t*-tests (2-tailed) comparing the experimental groups (*N*=9-10/group) across selected ROIs. Data from the contrasts specified by SPM were extracted by using inclusive three-dimensional ROI masks. Significant (*P*<0.05) differences between the groups are emphasized by bold lettering.

ROI	Contrast							
	Alcohol > water		Abstinence > water		Naltrexone > water		Alcohol > naltrexone	
	<i>t</i>	<i>p</i>	<i>t</i>	<i>p</i>	<i>t</i>	<i>p</i>	<i>t</i>	<i>p</i>
Orbital cortex	1.64	0.121	-2.39	0.031	-2.08	0.054	3.61	0.002
Prefrontal cortex	0.31	0.754	-2.42	0.029	-1.47	0.159	1.75	0.098
Insular cortex	4.42	0.000	-2.36	0.033	0.45	0.652	3.64	0.002
Primary/Secondary somatosensory cortex	6.63	0.000	-0.77	0.449	3.71	0.002	2.04	0.057
Primary/Secondary motor cortex	3.43	0.003	-0.14	0.888	1.38	0.185	1.94	0.069
Caudate putamen	0.83	0.416	-0.73	0.476	-1.37	0.188	2.53	0.021
Bed nucleus of the stria terminalis	0.57	0.573	-1.01	0.331	-0.33	0.744	0.78	0.441
Globus pallidus	1.52	0.149	-0.15	0.879	-0.05	0.954	1.98	0.065
Ventral pallidum	1.22	0.241	-0.38	0.703	-0.62	0.542	1.78	0.092
Amygdala	3.92	0.001	2.85	0.011	1.84	0.085	3.01	0.009
Lateral hypothalamus	-0.32	0.746	-0.02	0.981	-0.32	0.747	0.05	0.960
Zona incerta	4.03	0.001	0.55	0.588	1.65	0.119	1.95	0.068
Substantia nigra	0.38	0.705	1.00	0.333	0.55	0.587	-0.22	0.821
Ventral tegmental area	-0.11	0.908	0.43	0.672	-0.38	0.708	0.36	0.719
Pedunculopontine tegmental nucleus	3.54	0.002	1.95	0.070	3.80	0.001	-0.82	0.419

Figure 4 Twenty-four hour consumption of 10% alcohol (A) and water (B) in alcohol-drinking rats pretreated with saline or naltrexone (3 mg/kg). The data are expressed as mean $\pm$ SEM in groups of 9-10 subjects. **P*<0.05, ****P*<0.0001, paired *t*-test.

P=0.31). It is also to be noted that the 24-h measuring interval may not optimally reveal the effects of naltrexone on alcohol drinking, because rats will probably be able to compensate for the initial suppression of intake by naltrexone during the rest of their active period. In the water-drinking and alcohol-abstinent groups, no significant alterations in water consumption were seen during the 24-h interval (water group, $t_8=1.23$, *P*=0.25; abstinent group, $t_9=2.14$, *P*=0.06).

Figure 5 shows the depiction of the strength of the assumed monosynaptic connections between the nucleus accumbens and various brain regions derived from the within-group analysis and expressed as the percentage of activated voxels (*P*<0.0001) at each brain area. To a large extent, this diagram summarizes the findings from the statistical parametric maps shown in Figure 3.

Thus, water-drinking and abstinent rats displayed virtually identical connection strengths, with minor reductions in abstinent rats. However, compared to water-drinking rats, ongoing alcohol consumption enhanced activation in all regions connected to the nucleus accumbens, whereas the acute naltrexone injection suppressed activation universally.

3. Discussion

In the present experiments, we used MEMRI for characterization of the role of nucleus accumbens connections in the regulation of alcohol drinking and the suppression thereof by the nonselective opioid antagonist naltrexone. For this purpose, MnCl₂ was injected into the nucleus accumbens

Figure 5 Connectivity between the nucleus accumbens and brain regions with presumed monosynaptic connections with the accumbens. The thickness of the connecting lines expresses connection strength that was derived directly from the percentage of significant voxels ($P < 0.0001$, uncorrected) in three-dimensional ROIs identified on the basis of the statistical maps depicted in Figure 3. Abbreviations used for brain regions: VP, ventral pallidum; BST, bed nucleus of stria terminalis; CPu, caudate putamen; GP, globus pallidus; INS, insular cortex; LH, lateral hypothalamus; O, orbital cortex; PFC, prefrontal cortex; SN, substantia nigra; ZI, zona incerta. Prefrontal cortex (PFC) is a composite ROI encompassing the anterior cingulate, prelimbic, and infralimbic cortex.

and the global T1-weighted scans acquired immediately after the MnCl_2 injections were contrasted with those acquired 24 h later. This comparison revealed a clearly defined anatomical pathway from the nucleus accumbens to the substantia nigra and lateral VTA. Compared with the water-drinking rats, alcohol drinking increased signal intensity along this continuum without affecting intensity in the substantia nigra or VTA, whereas abstinent rats with a history of alcohol drinking displayed slightly decreased intensity. In contrast, a single naltrexone administration clearly suppressed alcohol-induced signal increase along this forebrain-midbrain tract. Because there were no differences between the experimental groups in the extension nor the amount of injected MnCl_2 , the observed differences in the Mn^{2+} accumulation cannot be explained by passive diffusion from the injection site, but were rather produced by differences in neural activity.

Tracing of specific neuronal pathways with MEMRI is based on the entry of Mn^{2+} into excitable neurons and the subsequent active axonal transport of Mn^{2+} , resulting in enhanced T1 signal in the axons of the pathway. The rostral-

caudal pathway revealed by the accumbal MnCl_2 injections in the present experiments is compatible with mapping of monosynaptic inputs using different techniques, such as rabies-virus-based transynaptic retrograde tracing from the substantia nigra and VTA (Watabe-Uchida et al., 2012). In addition to direct neural connections from the nucleus accumbens to the midbrain, our data also included the previously described descending projections from the accumbens core and shell to the ventral pallidum, globus pallidus, and lateral hypothalamus (Nakano, 2000). The majority of these feedback projections are GABAergic (Kalivas et al., 1993; Walaas and Fonnum, 1980), but the bed nucleus of the stria terminalis, preoptic area, and lateral hypothalamus also provide glutamatergic afferents into the substantia nigra/VTA (Geisler et al., 2007). Most probably, the connectivity of accumbal neurons with the above anatomical regions was established by anterograde tracing that is based on axonal transport mediated by kinesin. The present data also showed accumbal connectivity with regions of the neocortex, such as insula, orbito-frontal cortex, and the medial prefrontal cortex that are

known to send glutamatergic inputs to striatal regions (Noori et al., 2012). In principle, anterograde tracing of these glutamatergic tracts could only be achieved by transsynaptic relaying through thalamic nuclei (Hoover and Vertes, 2007). Indeed, Mn^{2+} ion transport was demonstrated to reach cortical areas across three synapses following striatal injections in primates (Murayama et al., 2006). In the present study, however, anterograde tracing of the prefrontal areas does not appear very likely given the high signal intensity in the neocortical areas and the low efficiency of polysynaptic Mn^{2+} transport (Canals et al., 2008), and therefore also the possibility of retrograde transport has to be considered. It has been shown that Mn^{2+} is also transported in a retrograde manner by dynein and retrograde kinesins (Matsuda et al., 2010), and previously intra-striatal $MnCl_2$ injections were demonstrated to increase signal intensity in the prefrontal cortex (Pautler et al., 2003). However, the kinetics and mechanisms of retrograde Mn^{2+} transport compared to the anterograde transport are not fully understood.

Stimulation of the striatal GABAergic medium spiny neurons expressing dopamine D1 receptors exerts inhibitory action on substantia nigra/VTA GABAergic neurons, thereby disinhibiting midbrain dopamine neurons (Kravitz et al., 2012). The selective activation of this direct pathway, with a modulatory role of the collaterals of the indirect pathway, is possibly the critical neural process underlying reinforcement (Lobb et al., 2011). Our present data indicating that alcohol drinking enhanced accumulation of Mn^{2+} in the rostral-caudal pathway visualized by accumbal Mn^{2+} injections is compatible with this view. Most likely, activation of the striatal medium spiny neurons caused influx of Mn^{2+} ions that were subsequently transported along axons to the midbrain, resulting in enhanced T1 signal that was retained in the pathway until subsequent imaging due to the slow efflux of Mn^{2+} ions. Indirectly, this enhancement implies alcohol-induced dopamine release to activate the medium spiny neuron D1 receptors, which is probably also expressed as alcohol-induced motor activation seen previously (Päiväranta and Korpi, 1993). In addition, we also saw signal enhancement by alcohol in neocortical regions that do not receive major direct inputs from the nucleus accumbens, but instead provide the nucleus accumbens with glutamatergic afferents. Signal enhancement in these regions would therefore depend on alcohol-induced activity of the glutamate terminals in the nucleus accumbens and subsequent retrograde Mn^{2+} transport. These neocortical regions included the insular cortex that is involved both in motivational processes driving or inhibiting addictive behavior and in interoceptive processes representing bodily states associated with drug effects (Kobayashi, 2011; Naqvi et al., 2014). In humans and rodents, the insula has previously been shown to be activated either by alcohol or alcohol-associated cues (Claus et al., 2011; Dudek et al., 2014). In the hindbrain, alcohol-induced activation emerged in the pedunculopontine tegmental nucleus that receives projections from the ventral pallidum and extended amygdala, possibly also from the nucleus accumbens (Zahm et al., 2001). The pedunculopontine tegmental nucleus provides excitatory input to midbrain dopamine neurons and has been suggested to be involved in reward-related behavior (Winn, 2006). As the Mn^{2+} injections were given both to the

core and shell regions of the nucleus accumbens, the activity of both compartments could contribute to the observed connectivity changes. The accumbens core and shell have distinct functional circuits, as well as overlap in their midbrain targets (Nakano, 2000). They have also been shown to have partially different roles in mediation of acute alcohol reward and seeking. Rats self-infused alcohol in the shell, but not in the core (Engleman et al., 2009), and alcohol seeking was reported to depend on the shell D1 receptors (Hamlin et al., 2007; Hauser et al., 2015). On the other hand, alcohol-associated cues increased *c-fos* expression in both compartments (Dayas et al., 2007). It is possible that the core mainly mediates alcohol seeking maintained by discrete cues, whereas the shell is involved in context-dependent alcohol seeking (Chaudhri et al., 2010).

In contrast to alcohol-induced activation of the accumbal projections, suppressed activity was seen in the orbital, insular, and prefrontal cortex in rats with a weeklong abstinence following long-term alcohol drinking. The present imaging method does not allow analysis of the circuitry or neurochemistry behind this finding, but insofar as the decreased signal intensity in these areas reflects activity-dependent uptake and retrograde transport of Mn^{2+} , the data implies attenuated activity of the cortical glutamatergic terminals in the nucleus accumbens. We also saw increased activity in the amygdala of abstinent rats. Together, these findings are intriguing in the light of the previously suggested functional disconnection of the medial prefrontal cortex and amygdala following abstinence from intermittent alcohol drinking (George et al., 2012).

Because naltrexone was given systemically, decreased alcohol-induced signal intensity in the accumbal output connections could result from opioid antagonist actions on multiple brain sites. As shown recently, intra-VTA naltrexone attenuated dopamine release in the nucleus accumbens, which could be due to opioid antagonist-mediated blockade of disinhibition of the dopaminergic cell bodies in the VTA (Valenta et al., 2013). However, there is also evidence that locally applied naltrexone or naltrindole could attenuate alcohol-induced dopamine release in the nucleus accumbens (Acquas et al., 1993; Benjamin et al., 1993), which is consistent with recent findings that the μ -opioid receptors in the accumbal direct-pathway medium spiny neurons regulate midbrain dopamine neurons and mediate opiate reinforcement (Cui et al., 2014). Moreover, decreased alcohol reinforcement following intra-amygdala opioid antagonist administration suggests a role for amygdalar opioid receptors in the effects of systemic opioid antagonists (Heyser et al., 1999). Collectively, these data may point to naltrexone's actions on multiple sites in a network regulating the activity of the accumbal connections, and therefore more work for clarifying naltrexone's mode of action is warranted.

An important question for understanding the interaction between naltrexone and alcohol drinking is whether the reductions in the nucleus accumbens connectivity were caused solely by decreased alcohol consumption by acute naltrexone or whether naltrexone would suppress neuronal activity in the absence of alcohol stimulation. As the effects of naltrexone alone were not assessed, this question remains without a definite answer. However, there are data to suggest that the opioidergic tone in the nucleus

accumbens is negligible under basal conditions. For example, naltrexone doses comparable to the one in the present study did not alter the accumbal dialysate dopamine concentrations in the absence of drug-induced stimulation (Gonzales and Weiss, 1998; Piepponen et al., 1999; Valenta et al., 2013), and naltrexone alone did not alter the threshold of brain stimulation reward over a wide range of doses (Easterling and Holtzman, 2004; Todtenkopf et al., 2009). According to neural activity measurement using *c-fos* expression in the nucleus accumbens, naltrexone may even be disinhibitory (Days et al., 2007). Although these data suggest that naltrexone does not acutely affect basal neuronal activity in the nucleus accumbens or the brain reward circuitry in general, we cannot exclude the possibility that naltrexone alone has also long-term effects during the 24-h imaging interval. In addition, the current data did not allow a detailed temporal analysis of the relationship between the observed MEMRI activity pattern and the behavioral output, i.e., alcohol drinking. These remaining questions notwithstanding, the parallels of our findings with human neuroimaging, such as the suppression of alcohol cue-induced activation of the ventral striatum and orbito-frontal cortex by naltrexone (Myrick et al., 2008; Schacht et al., 2013b), reinforce the idea that imaging-based biomarkers emerging from preclinical rodent models could be predictive of treatment effects in human alcoholics.

In summary, we aimed at elucidating the role of the nucleus accumbens connections in the regulation of alcohol drinking and its suppression by the nonselective opioid antagonist naltrexone. To this end, signal intensity resulting from the intra-accumbal MnCl₂ injection was determined from T1 scans acquired 1 and 24 h postinjection. This comparison revealed a rostral-caudal pathway that is compatible with the established anatomy of the accumbal output projections. Alcohol drinking activated this pathway, as well as prefrontal connections, whereas acute naltrexone suppressed them. These data suggest that mapping the effects of pharmacological agents in discrete anatomically defined pathways could be a useful translational tool for developing and evaluating pharmacotherapies for alcohol use disorders.

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The funding agencies had no role in the study design, collection, analysis and interpretation of data, writing of the report, or the decision to submit the paper for publication.

Contributors

Mateusz Dudek and Petri Hyttiä designed and performed the experiments. Mateusz Dudek wrote the first draft of the manuscript. Santiago Canals and Wolfgang Sommer contributed to the data analysis and presentation, and provided critical revisions of the manuscript. Petri Hyttiä edited the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors have no potential conflicts of interest to declare in relation to the work described.

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