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RAPID COMMUNICATION

CINP

Orexin-1 receptor signalling within the ventral tegmental area, but not the paraventricular thalamus, is critical to regulating cue-induced reinstatement of cocaine-seeking

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Abstract

Orexinergic signalling is critical to drug relapse-like behaviour; however, the CNS sites(s) of action remain unknown. Two candidate brain regions are the paraventricular thalamus (PVT) and ventral tegmental area (VTA). We assessed the effect of intra-PVT or -VTA administration of the orexin-1 receptor (OrxR1) antagonist SB-334867 on discriminative cue-induced cocaine-seeking. Animals received either PVT- or VTA-directed SB-334867 (0, 3 or 6 μg; 0, 1 or 3 μg, respectively) prior to reinstatement testing elicited by presenting cocaine-paired stimuli (S^+). The effect of VTA-directed injections of SB-334867 (0 or 3 μg) on locomotor activity was also assessed. Intra-VTA, but not -PVT, SB-334867 dose-dependently attenuated S^+-induced reinstatement (3 μg dose, $p<0.01$). Intra-VTA SB-334867 had no effect on locomotor activity. We conclude that OrxR1 signalling within the VTA, but not the PVT, mediates cue-induced cocaine-seeking behaviour. We hypothesize that blockade of VTA OrxR1 signalling may reduce nucleus accumbens dopamine in response to drug cue presentation.

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Key words: Cocaine, orexin, paraventricular thalamus, relapse, ventral tegmental area.

Introduction

Orexin (hypocretin) is a peptide neurotransmitter that is expressed in a discrete population of cells within the posterior lateral hypothalamus (de Lecea *et al*. 1998; Sakurai *et al*. 1998). Neurons expressing orexin project to a number of reward-associated structures within the brain indicating a likely role for these neurons in reward function and motivation. Indeed, early studies implicated orexin in the regulation of appetite and food-seeking (Sakurai, 1999). More recently, orexin has been implicated in addiction (Aston-Jones *et al*. 2010) with several demonstrations that this neuropeptide plays a role in regulating the reinforcing and rewarding effects of highly salient rewards including high-fat food and some drugs of abuse (Borgland *et al*. 2009; Boutrel *et al*. 2005; Hollander *et al*. 2008; Lawrence *et al*. 2006).

With respect to drug-seeking, Harris and colleagues (2005) demonstrated that hypothalamic orexin cells are activated by cues previously associated with cocaine and morphine availability and that systemic administration of the orexin-1 receptor (OrxR1) antagonist, SB-334867, blocks reinstatement of morphine place preference. Systemic administration of SB-334867 also blocks both cue- and stress-induced reinstatement (Boutrel *et al*. 2005; Lawrence *et al*. 2006; Smith *et al*. 2009). At present, however, the specific site(s) of relapse-related orexin signalling within the brain remains unclear.

We, among others, have recently suggested that the paraventricular thalamus (PVT) might be a potential integrator of hypothalamic peptide signals and responsible for relaying reward-relevant information to brain regions that trigger drug-seeking (Dayas *et al*. 2008; Hamlin *et al*. 2009; Kelley *et al*. 2005). Indeed, the

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PVT has been shown to receive input from hypothalamic orexin neurons (Parsons *et al*. 2006; Peyron *et al*. 1998) and PVT neurons send projections that innervate the nucleus accumbens (NAc) shell (Hamlin *et al*. 2009). Further, inactivation of the PVT blocks both cue- and drug-primed reinstatement (Hamlin *et al*. 2009; James *et al*. 2010) and presentation of cues previously associated with drug availability activate PVT neurons that are closely apposed to hypothalamic orexin-positive terminals (Dayas *et al*. 2008). At present, however, it is unclear whether disruption of orexin signalling within the PVT modulates reinstatement of drug-seeking behaviour.

Another brain region through which orexin might mediate its pro-reward-seeking effects is the ventral tegmental area (VTA). The VTA has been shown to express both orexin receptor subtypes (OrxR1 and OrxR2) (Marcus *et al*. 2001; Narita *et al*. 2006) and electrophysiological studies have shown that orexin applied to midbrain slices increases VTA dopamine neuron excitability (Korotkova *et al*. 2003). Although anatomical studies demonstrate that lateral hypothalamus orexin neurons do not make frequent synaptic contacts within the VTA (Balcita-Pedicino & Sesack, 2007), intra-VTA orexin stimulates local dopamine and glutamate release, which is suggested to indirectly activate dopamine neurons by local paracrine diffusion or volume transmission (Wang *et al*. 2009). Importantly, dopaminergic projections from the VTA to regions such as the NAc are known to play a key role in mediating reinstatement of drug-seeking behaviour (Bossert *et al*. 2007). Interestingly, intra-VTA infusion of orexin-A into the VTA reinstates extinguished preference for a morphine-paired environment (Harris *et al*. 2005); however, intra-VTA infusion of an OrxR1 antagonist does not block stress-induced reinstatement (Wang *et al*. 2009). It remains to be determined whether OrxR1 signalling within the VTA plays a role in mediating cue-induced relapse in a self-administration paradigm.

In the present study, we tested whether infusion of the OrxR1 antagonist SB-334867 into either the PVT or VTA modified reinstatement of drug-seeking elicited by discriminative cues previously associated with cocaine availability. Our results demonstrate a role for OrxR1 signalling in the VTA but not the PVT in cocaine-seeking behaviour that is not a consequence of non-specific effects on locomotor activity.

Methods

Ethical statement

All procedures performed were approved by the University of Newcastle Animal Care and Ethics Committee and were performed in accordance with the New South Wales Animal Research Act.

Drugs

Cocaine hydrochloride (Johnson Matthey, UK) was dissolved in sterile physiological saline (2.5 mg/ml). Similar to a number of previous studies (Li *et al*. 2010*a*,*b*, 2011), SB-334867 was dissolved in 100% DMSO due to difficulties dissolving this compound in lower DMSO concentrations. Importantly, avoidance memory studies report that 100% DMSO does not alter the antagonist properties of the SB compound, nor does it produce behavioural effects compared to saline (Akbari *et al*. 2006, 2007, 2008). Similarly, 100% DMSO has been used as a vehicle in the VTA without consequence (Pierce *et al*. 1999). In addition, pilot studies revealed no differences between vehicle (DMSO) and no-injection controls in terms of reinstatement behaviour in animals implanted with PVT-directed guide cannulae (see Fig. S1, online). As we and others (e.g. Hamlin *et al*. 2009; James *et al*. 2010) have previously demonstrated that the PVT is critical to reinstatement, any functional effects of 100% DMSO on reinstatement behaviour would be revealed by comparing PVT-DMSO injections with no-injection controls.

Animals and surgery

Male Sprague–Dawley rats weighing 200–250 g upon arrival (Animal Resources Centre, Australia) were housed two per cage on a reversed 12-h light/dark cycle (lights off 07:00 hours) with food and water available *ad libitum*. Rats were handled daily for 1 wk before undergoing intravenous catheter surgery as described previously (James *et al*. 2010). To facilitate the placement of PVT- or VTA-directed guide cannulae, animals were anaesthetized with isofluorane and then placed in a sterotaxic frame (Stoelting, USA). Craniotomies were made into the skull and stainless-steel guide cannulae (26-gauge, Small Parts, USA) lowered unilateraly to the level of either the VTA (AP −5.3, ML ±0.8, DV −8.0) or PVT (AP −2.6, ML 0.0, DV −4.6; Paxinos & Watson, 2007). Guide cannulae were secured to four stainless-steel jewellers' screws (Mann Optics, Australia) with dental cement (Henry Schein, Australia) and were kept clear by stainless-steel stylets (33-gauge, Small Parts, USA).

Expt 1: effect of intra-PVT or -VTA SB-334867 administration on cue-induced reinstatement

Self-administration training

Seven days after catheter surgery, rats were trained to self-administer cocaine hydrochloride (0.25 mg/

injection over 4 s) intravenously (i.v.) in 3-h sessions, once daily/5 d a week. Training was continued until stable responding for cocaine was achieved (±10% over three sessions) at which time animals were subjected to daily, 2-h randomized conditioning sessions for cocaine or saline infusions (FR1), in the presence of distinct discriminative stimuli. For cocaine, this involved a constant 70-dB white noise and illumination of the white cue-light (20 s) above the active lever (S^+) and for saline, constant illumination of the white house-light and a 20-s intermittent beeping tone (S^-). Responding on the inactive lever was recorded but did not result in cocaine or saline infusions. After 16 d of conditioning (8 S^+/8 S^- sessions) lever responding was extinguished in daily, 1-h sessions until stable responding ($\leqslant$6 responses per session over three consecutive days) was achieved. During extinction, discriminative stimuli were withheld along with i.v. cocaine infusions. Four animals were excluded from the study due to catheter failure during the training period.

Reinstatement testing

After extinction, animals were presented with the S^- cue and tested for reinstatement. On the following day, animals received either PVT-directed SB-334867 (3 μg/0.5 μl, $n=7$; 6 μg/0.5 μl, $n=8$), VTA-directed SB-334867 (1 μg/0.5 μl, $n=6$; 3 μg/0.5 μl, $n=7$) or vehicle (100% DMSO; VTA, $n=6$; PVT; $n=8$). Fifteen minutes post-injection animals were tested for reinstatement under S^+ conditions for 1 h. One hour following the end of testing, animals received either an intra -PVT or -VTA injection of Cresyl Violet (0.5 μl) using the same injector that was used to infuse either the SB-334867 or vehicle, before being perfused. Brains were removed and later sectioned on a freezing microtome (Leica SM 2000R, Leica Biosystems, Germany). Injection sites for each animal were verified by inspecting a series of 40-μm (PVT) or 50-μm (VTA) tissue slices surrounding the injection site under a light microscope (CX40, Japan) at 40x magnification.

Expt 2: effect of intra-VTA SB-334867 administration on locomotor activity

Animals ($n=8$) were trained to self-administer cocaine in the manner described in expt 1. Following discriminative cue-training, animals were habituated to black circular arenas (40 cm diameter, 39 cm height) in 1-h sessions over 3 d. On the final day of habituation, locomotor activity was recorded to provide a baseline measure of activity. One day later, animals received either an intra-VTA infusion of SB-334867 (3 μg/0.5 μl; $n=4$) or DMSO ($n=4$) and locomotor activity was assessed for 1 h. Locomotor activity was subsequently analysed using EthoVision XT (Noldus Information Technology, USA).

Data analysis

Within both PVT- and VTA-guide implanted cohorts, single-factor ANOVAs were used to compare treatment groups for responding during conditioning, overall cocaine intake and time taken to reach extinction. For PVT-guide implanted animals, differences in reinstatement were assessed using a 2-session (extinction, reinstatement) × 3-treatment (vehicle, 3 μg SB-334867, 6 μg SB-334867) mixed-model ANOVA. Similarly, reinstatement was assessed in VTA-guide implanted animals using a 2-session (extinction, reinstatement) × 3-treatment (vehicle, 1 μg SB-334867, 3 μg SB-334867) mixed-model ANOVA. Where significant interactions were observed, Tukey's *post-hoc* tests were used to assess differences between treatment groups. To assess differences in locomotor activity on both days of testing, 2-treatment (DMSO, 3 μg SB-334867) × 6-time (10, 20, 30, 40, 50, 60 min) mixed-model ANOVAs were performed. Animals that received misdirected guide cannulae placements into either the PVT or VTA were excluded from the analysis.

Results

One animal received a misdirected PVT injection of the 6 μg dose of the SB-334867 compound that was targeted at the mediodorsal thalamic nucleus and was excluded from the analysis (see Fig. S2, online). All other PVT-directed injectors were localized to the PVT or the boundary of the PVT and intermediodorsal thalamus. Importantly, reinstatement did not differ between centrally *vs.* ventrally placed PVT injections (see Fig. S2, online). All VTA injections were accurate, with the majority falling into the paranigral nucleus and parabrachial pigmented nucleus of the caudal extent of the VTA (see Fig. S2, online).

Within each cohort (i.e. either PVT- or VTA-guide implanted animals) vehicle- or SB-334867-treated animals did not differ in terms of overall cocaine consumption, responding under S^- and S^+ training conditions or time taken to reach extinction (see Training Statistics section, available online). It should be noted, however, that animals prepared with PVT-directed guide cannulae exhibited lower levels of cocaine-reinforced responding during conditioning compared to VTA-guide implanted animals. This was associated with an overall lower level of reinstatement

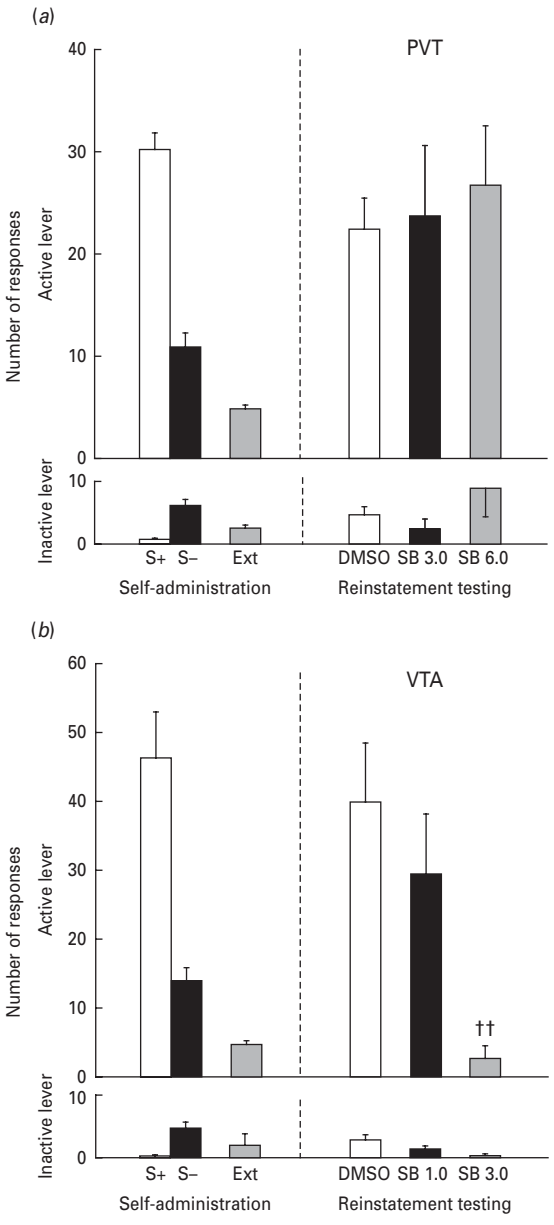

Fig. 1. (*a*) Intra-PVT administration of either 3 μg or 6 μg of SB-334867 had no effect on responding on either the active or inactive lever following presentation of the S^+ cue. S^+, Average number of responses for all treatment groups over the final three S^+ conditioning trials. This level of responding resulted in an average of 26.61 ($\pm$1.20) cocaine infusions per session. S^-, Average number of responses for all treatment groups over the final three S^- conditioning trials. This level of responding resulted in an average of 7.05 ($\pm$0.92) saline infusions per session. (*b*). Intra-VTA administration of SB-334867 dose-dependently attenuated cue-induced reinstatement of cocaine-seeking behaviour, with the 3 μg dose reducing responding to extinction levels. Treatment had no significant effect on inactive lever responding. †† $p<0.01$ compared to controls. S^+, Average number of responses for all treatment groups over the final three S^+ conditioning trials. This level of responding resulted in an average of 32.23 ($\pm$1.58) cocaine infusions per session. S^-, Average number of responses for all treatment groups over the final three S^- conditioning trials. This level of responding resulted in an average of 8.25 ($\pm$0.92) saline infusions per session. Ext, Average number of responses for all treatment groups over the final three extinction sessions.

following the presentation of the S^+ stimuli. This is likely to be due to a cohort effect, as animals were trained and tested separately and at different times of the year.

Effect of PVT-directed SB-334867 on S^+-induced reinstatement

A significant main effect of session revealed an overall increase in responding on the active lever following presentation of the S^+ cue ($F_{1,19}=43.02$, $p<0.001$). Importantly, no session $\times$ treatment interaction was observed ($p=0.73$) indicating that neither dose of SB-334867 administered into the PVT affected reinstatement compared to vehicle treatment (Fig. 1). Similarly, responding on the inactive lever did not differ between groups ($p=0.20$).

Effect of VTA-directed SB-334867 on S^+-induced reinstatement

A significant main effect of session indicated that presentation of the S^+ cue produced an overall reinstatement of responding on the active lever ($F_{1,16}=23.34$, $p<0.001$). A significant session $\times$ treatment interaction was also observed, suggesting differences in the extent to which treatment affected S^+-elicited reinstatement ($F_{2,16}=7.80$, $p<0.01$). *Post-hoc* analyses revealed that intra-VTA treatment with SB-334867 dose-dependently attenuated reinstatement, with the 3 μg dose producing a significant reduction compared to controls ($p<0.01$, Fig. 1). Importantly, SB-334867 treatment had no effect on inactive lever responding ($p=0.22$).

Effect of VTA-directed SB-334867 on locomotor activity

There was no statistical difference between treatment groups at any time-point during baseline recording ($p=0.57$). On test day, intra-VTA infusion of SB-334867 had no effect on locomotor activity compared to DMSO-treated controls at all time-points tested ($p=0.77$, Fig. 2).

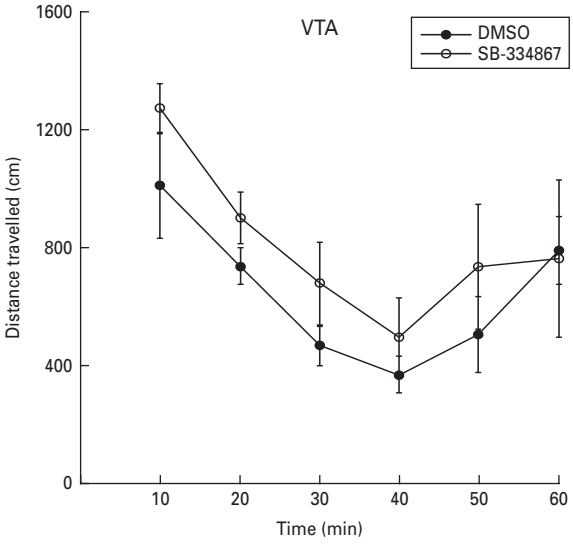

Fig. 2. Animals that received intra-VTA infusions of SB-334867 (3 μg/0.5 μl) did not differ from vehicle-treated controls in terms of locomotor activity across the entire 1-h period.

Discussion

The present findings demonstrate, for the first time, that infusion of the OrxR1 antagonist SB-334867 directly into the VTA attenuates reinstatement of cocaine-seeking behaviour elicited by a cocaine S^+ previously associated with drug availability. In contrast, infusion of SB-334867 into the PVT had no effect on cue-induced reinstatement. These findings build upon previous evidence that central or peripheral administration of OrxR-1 antagonists block both cue- and stress-induced reinstatement of drug-seeking (Boutrel *et al*. 2005; Lawrence *et al*. 2006; Smith *et al*. 2009).

We have previously suggested that the PVT may influence reinstatement of drug-seeking; a hypothesis based on several experimental findings. First, inactivation of the PVT attenuates context- and drug-induced reinstatement of alcohol- and cocaine-seeking, respectively (Hamlin *et al*. 2009; James *et al*. 2010). Furthermore, Hamlin and colleagues (2009) demonstrated that the percentage of PVT neurons that demonstrate Fos-like immunoreactivity and project to the NAc shell increases after context-induced renewal of alcohol-seeking. There is evidence to suggest that these effects may be mediated by orexin signalling, as the PVT is densely innervated by orexin neurons (Peyron *et al*. 1998) and orexin terminals make putative contact with PVT neurons that project to the NAc shell (Parsons *et al*. 2006). Furthermore, drug cue-sensitive PVT neurons are closely apposed to orexin-positive neuronal terminals (Dayas *et al*. 2008). Thus, the present demonstration of a lack of involvement of PVT OrxR1 signalling in reinstatement-like behaviour is somewhat unexpected.

It should be noted that a role for PVT orexin release in drug-seeking cannot be completely ruled out, as the PVT is also known to strongly express OrxR2 (Marcus *et al*. 2001). Of relevance here is evidence that orexin-B produces stronger actions on PVT neurons than orexin-A (Huang *et al*. 2006). Furthermore, intra-PVT administration of OrxR2-specific antagonists have been recently shown to decrease footshock-evoked anxiety-like behaviour (Li *et al*. 2010*b*), suggesting a possible role for PVT OrxR2 signalling in stress-induced reinstatement. It will therefore be important for future experiments to determine whether PVT-directed injections of compounds that specifically modulate OrxR2 signalling alter relapse-like behaviour.

We also investigated whether VTA-directed injections of the OrxR1 antagonist alter cue-induced reinstatement and show an important role for this receptor subtype in cocaine-motivated responding. Early electrophysiological studies demonstrated that intra-VTA administration of orexin produces an increase in the firing frequency of VTA dopaminergic (and non-dopaminergic) neurons (Korotkova *et al*. 2003). While orexin terminals were later shown to make infrequent contact within the VTA (Balcita-Pedicino & Sesack, 2007), both OrxR1 and OrxR2 have been identified on the surface of VTA dopamine cells (Narita *et al*. 2006). Furthermore, in VTA neurons, orexin-mediated potentiation of NMDA receptor (NMDAR) excitatory post-synaptic currents was increased in animals trained to self-administer cocaine and high-fat food over animals trained to respond for regular food (Borgland *et al*. 2009). This change was linked to increased presynaptic glutamate release. An earlier study from this group identified that orexin can also potentiate NMDAR currents through protein kinase C-dependent trafficking of post-synaptic NMDARs (Borgland *et al*. 2006), indicating that orexin may alter the regulation of VTA dopamine neuron activity through both pre- and post-synaptic mechanisms. Finally, using microdialysis, Wang and colleagues (2009) showed that intra-VTA infusion of orexin increases local dopamine and glutamate levels.

Consistent with electrophysiological data, behavioural studies have reported that infusion of orexin directly into the VTA produces reinstatement of extinguished morphine and cocaine preference (Harris *et al*. 2005; Wang *et al*. 2009). In addition, unilateral intra-VTA SB-334867 (10 nmol) administration reduces

morphine-induced place preference (Narita *et al*. 2006). In the present study we extend upon these findings and show for the first time that OrxR1 signalling within the VTA mediates cocaine S^+-induced relapse. Although not directly addressed in the present study, a parsimonious explanation for this effect is that antagonism of OrxR1 decreased dopamine release within the NAc (Narita *et al*. 2006). Indeed, intra-VTA orexin administration is associated with increased dopamine release in the NAc (Narita *et al*. 2006) and context-induced reinstatement is sensitive to dopamine receptor blockade in this brain region (Bossert *et al*. 2007).

Interestingly, the effective dose in the current study (3 μg or 9.4 nmol) was lower than that published in two other behavioral studies. Zheng *et al*. (2007) showed that bilateral intra-VTA infusion of SB-334867 (15 nmol/side) was required to reduce high-fat food consumption, while Hollander *et al*. (2008) report that bilateral intra-insular infusions of 1 μg and 5 μg (6.2 and 31.2 nmol, respectively) doses of SB-334867 attenuated nicotine self-administration. The robust effects observed in our study by unilateral VTA injections of a lower dose may indicate some spread of injectate to the contralateral VTA and/or differences in the dose of antagonist that is required to suppress behaviour elicited by primary (Hollander *et al*. 2008; Narita *et al*. 2006; Zheng *et al*. 2007) *vs.* conditioned reinforcers.

Importantly, intra-VTA SB-334867 did not reduce general locomotor activity in animals that were trained to self-administer cocaine. This was despite an apparent, but statistically non-significant, decrease in inactive lever responses in SB-334867-treated animals. The lack of a non-specific effect of SB-334867 is consistent with other studies showing that intra-VTA infusions of the OrxR1 antagonist did not alter locomotor activity (Borgland *et al*. 2006). It is plausible that the trend towards decreased inactive lever responding is actually due to the effects of SB-334867 on drug-seeking behaviour.

In summary, we show for the first time that OrxR1 signalling within the VTA, but not the PVT, is critical in mediating cue-induced reinstatement. Taking previous findings into consideration, this effect is likely to be mediated by reduced activity of VTA dopamine neurons that target the NAc. Future studies will need to assess whether OrxR2 signalling within the PVT modulates drug-seeking. It is also important to note that orexin signalling may modulate reinstatement at other central sites including the insular (Hollander *et al*. 2008) and prefrontal (Jupp *et al*. 2010) cortex.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

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Statement of Interest

None.

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