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Short Communication

Direct effects of antipsychotic drugs on insulin, energy sensing and inflammatory pathways in hypothalamic mouse neurons

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ABSTRACT

Introduction: Second-generation antipsychotics cause serious metabolic side effects, but the mechanisms behind these effects remain largely unknown. However, emerging evidence supports that antipsychotics may act upon the hypothalamus, the primary brain region understood to regulate energy homeostasis. We have recently reported that the antipsychotics olanzapine, clozapine, and aripiprazole can directly act on hypothalamic rat neurons (rHypoE-19) to impair insulin, energy sensing, and modulate inflammatory pathways. In the current paper, we sought to replicate these findings to a mouse neuronal model.

Methods: The mouse hypothalamic neuronal cell line, mHypoE-46, was treated with olanzapine, clozapine, or aripiprazole. Western blots were used to measure the energy sensing protein AMPK, components of the insulin signalling pathway (AKT, GSK3β), and components of the MAPK pathway (ERK1/2, JNK, p38), the latter linked to inflammation. RT-qPCR was used to measure mRNA expression of the inflammatory mediators *IL-6*, *IL-10*, and *BDNF,* well as putative receptors in the mHypoE-46 (current) and the rHypoE-19 (previously studied) cell lines.

Results: In the mHypoE-46 neurons, olanzapine and aripiprazole increased AMPK phosphorylation, while clozapine and aripiprazole inhibited insulin-induced phosphorylation of AKT. Clozapine increased JNK and aripiprazole decreased ERK1/2 phosphorylation. Olanzapine also decreased *IL-6* mRNA expression, while olanzapine and clozapine increased *IL-10* mRNA expression. The rHypoE-19 neurons expressed the H_1, $5HT_{2A}$, and M_3 receptors, while the mHypoE-46 neurons expressed the $5HT_{2A}$, D_2, and M_3 receptors. Neither cell line expressed the $5HT_{2C}$ receptor.

Conclusion: Similar to observed effects of these agents in rat neurons, induction of AMPK by aripiprazole and olanzapine suggests impaired energy sensing, while suppression of insulin-induced pAKT by clozapine and aripiprazole suggests impaired insulin signalling, seen across both rodent derived hypothalamic cell lines. Conversely, olanzapine-induced suppression of pro-inflammatory *IL-6*, alongside olanzapine and clozapine-induced *IL-10*, demonstrate anti-inflammatory effects, which do not corroborate with our prior observations in the rat neuronal line. The different findings between cell lines could be explained by differential expression of neurotransmitters receptors and/or reflect genetic heterogeneity across the rat and mouse lines. However, overall, our findings support direct effects of antipsychotics to impact insulin, energy sensing, and inflammatory pathways in hypothalamic rodent neurons.

1. Introduction

Second-generation antipsychotics (APs) cause serious metabolic side effects, including type 2 diabetes and obesity, but the mechanism underlying these effects, particularly through the central nervous system (CNS), is largely unknown. APs are known to antagonize a range of neurotransmitter receptors, such as the dopamine-2 ($D_{2)}$ receptor, histamine-1 (H_1) receptor, muscarinic receptors, and serotonin (5HT) receptors, which contribute to the regulation of energy homeostasis. Emerging literature has implicated antagonism of these receptors in the

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metabolic side effects of APs (Kowalchuk et al., 2018). In addition, studies have suggested that APs can directly influence insulin signalling and inflammation in the CNS. Our lab recently showed that olanzapine, clozapine, and aripiprazole can directly regulate hypothalamic rat neurons. Specifically, we found that these agents can directly inhibit insulin signalling and energy sensing, and may upregulate inflammatory pathways (Kowalchuk et al., 2019). To explore the translatability of these findings, we sought to replicate these results in a mouse hypothalamic neuronal cell line. In addition, to explore mechanisms and understand potential similarities and differences between the rat and mouse hypothalamic neuronal lines, we screened the cell lines for expression of key neurotransmitter receptors that have previously been associated with metabolic side effects of APs.

2. Methods

Mouse hypothalamic neurons were immortalized using SV-40 T-antigen as previously described (Belsham et al., 2004). The immortalized mouse hypothalamic embryonic neuronal cell line, mHypoE-46, was selected as it has been previously characterized to express the insulin receptor and cytokine receptors. This cell line was also chosen as it is a neuropeptide Y (NPY) and agouti-related protein (AgRP) expressing embryonic lines that shares overlap in expression of insulin and inflammatory receptors (Belsham et al., 2004) with the rHypoE-19 neurons we used in our previously published work (Kowalchuk et al., 2019).

2.1. Antipsychotic treatment

In keeping with our previously published work in rHypoE-19 neurons, we treated the mHypoE-46 neurons with three second generation APs: clozapine, olanzapine, and aripiprazole. Olanzapine and clozapine were investigated as they have the highest associated clinical metabolic risk. Clozapine is additionally the only AP with superiority in treatment-resistant schizophrenia. Aripiprazole was selected as unlike most APs which antagonize the D_2 receptor, aripiprazole is a partial D_2 receptor agonist at lower clinical doses. It is also suggested to have a lesser clinical metabolic risk compared to other APs. Doses of each AP were initially chosen based on effects in the rHypoE-19 neurons (Kowalchuk et al., 2019). However, tolerated concentrations in the mHypoE-46 neurons were generally lower as compared to the previously studied rHypoE-19 neurons (Kowalchuk et al., 2019). The mHypoE-46 neurons were treated with olanzapine (100 & 20 u M), clozapine (1 & 10 u M), or aripiprazole (1 & 10 u M).

2.2. Experimental techniques

Methods for western blotting and RT-qPCR were performed as previously described (Kowalchuk et al., 2019). Prior to treatment for western blotting experiments, cells were serum starved for 1 h in low-glucose DMEM (MilliporeSigma, Oakville, ON, Canada) with added 1% penicillin, and treated with serum-free media. The blots were probed for phospho-AKT, AKT, phospho-AMPK, AMPK, phospho-p44/42 (ERK1/2), p44/42 (ERK1/2), phospho-JNK, JNK, phospho-P38, P38 (CST), phospho- GSK3β, and GSK3β (MilliporeSigma, Oakville, ON, Canada). Primers for the neurotransmitter receptors are as follows: H1R: F:CTGTTTCCGTCTCGACATCA, R:TGAGCCCCTGACATCTCC; D2R: F:CCACCACGGCCTACATAGCA, R:AATCTTGGCGTGCCCATTCT; 5HT2AR: F:GCTCTGTGCGATCTGGATTTA, R:TCAGGAAGGCTTTGGTTCTG; 5HT2CR: F:GATTGGACTGAGGGACGAAAG, R:CGCAGGACGTAGATCGTTAAG; M3R: F:CCTATTGGAATCTGGGCTACTG, R:CTCTGCTCCTACTGCTCTTT.

2.3. Assessment of cell viability

Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric cell viability assay (Denizot and Lang, 1986). Both the rHypoE-19 and mHypoE-46 cells were plated in 96-wells at 80% confluency and treated with olanzapine (100 & 20 μM, respectively), clozapine (20 & 10 μM, respectively), and aripiprazole (20 & 10 μM, respectively) for 24 h. Immediately after the treatment course, MTT powder was dissolved at a concentration of 5 mg/ml and incubated in the plate with phenol red-free media for 2 h. Each treatment was then evaluated in triplicate and the plates were read at 570 nm.

2.4. Statistical analysis

Data were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) and presented as mean ± SEM. One- or two-way ANOVA were used with Tukey's *post hoc* test. Statistical significance is considered as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

3. Results

3.1. Antipsychotics induce changes in insulin signalling and energy sensing in mHypoE-46 neurons

AKT and GSK3B are key components of the insulin signalling pathway, downstream of the insulin receptor. Phosphorylation of AKT results in AKT activation while phosphorylation of GSK3B inhibits GSK3B action. Cells were treated with the AP or vehicle with and without insulin to determine if each individual agent impairs insulin response. Clozapine (1 μM) and aripiprazole (10 μM) decreased insulin-stimulated pAKT, while olanzapine had no effect (Fig. 1A). None of the APs influenced pAKT protein expression in the absence of insulin co-treatment. In addition, none of the APs had an effect on GSK3β protein expression. AMPK was also investigated as, though not part of the canonical insulin signalling pathway, it is activated in times of low energy to regulate energy homeostasis. Olanzapine (100 μM) and aripiprazole (10 μM) significantly increased pAMPK (Fig. 1A/C), while clozapine had no effect on pAMPK protein expression (Fig. 1A) Table 1.

3.2. Antipsychotics induce changes in MAPKs and inflammatory mediators in mHypoE-46 neurons

3.2.1. MAPKs

Extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun amino-terminal kinases (JNK), and p38 are mitogen activated protein kinases (MAPKs). They are involved in various cell signalling responses, one of which is the cellular inflammatory response (Cargnello and Roux, 2011). After 15 min, clozapine (10 μM) treatment significantly increased pJNK, while aripiprazole (10 μM) decreased pERK1/2. Olanzapine had no effect on protein expression of any of the MAPKs (Fig. 1A) Table 1.

3.2.2. IL6, IL10, BDNF

The inflammatory mediators examined were chosen based on studies looking at cytokine changes in schizophrenia. IL6, IL10 (Maier et al., 2005), and BDNF (Pandya et al., 2013) have all been linked to the pathophysiology of schizophrenia as well as general metabolic regulation. After 4 h, olanzapine (20 μM) and clozapine (20 μM) increased *IL10* mRNA expression nearly 2- and 4- fold, respectively; this effect was no longer observed at 24 h. *IL6* mRNA expression was decreased compared to vehicle after 24 h of olanzapine (20 μM) treatment. Aripiprazole (10 μM) treatment had no effect on *IL6*, *IL10*, or *BDNF* mRNA levels at either time point (Fig. 1B) Table 1.

3.3. Cell viability

To determine whether toxicity associated with AP treatment could have played a role in the differences in cytokine changes between the

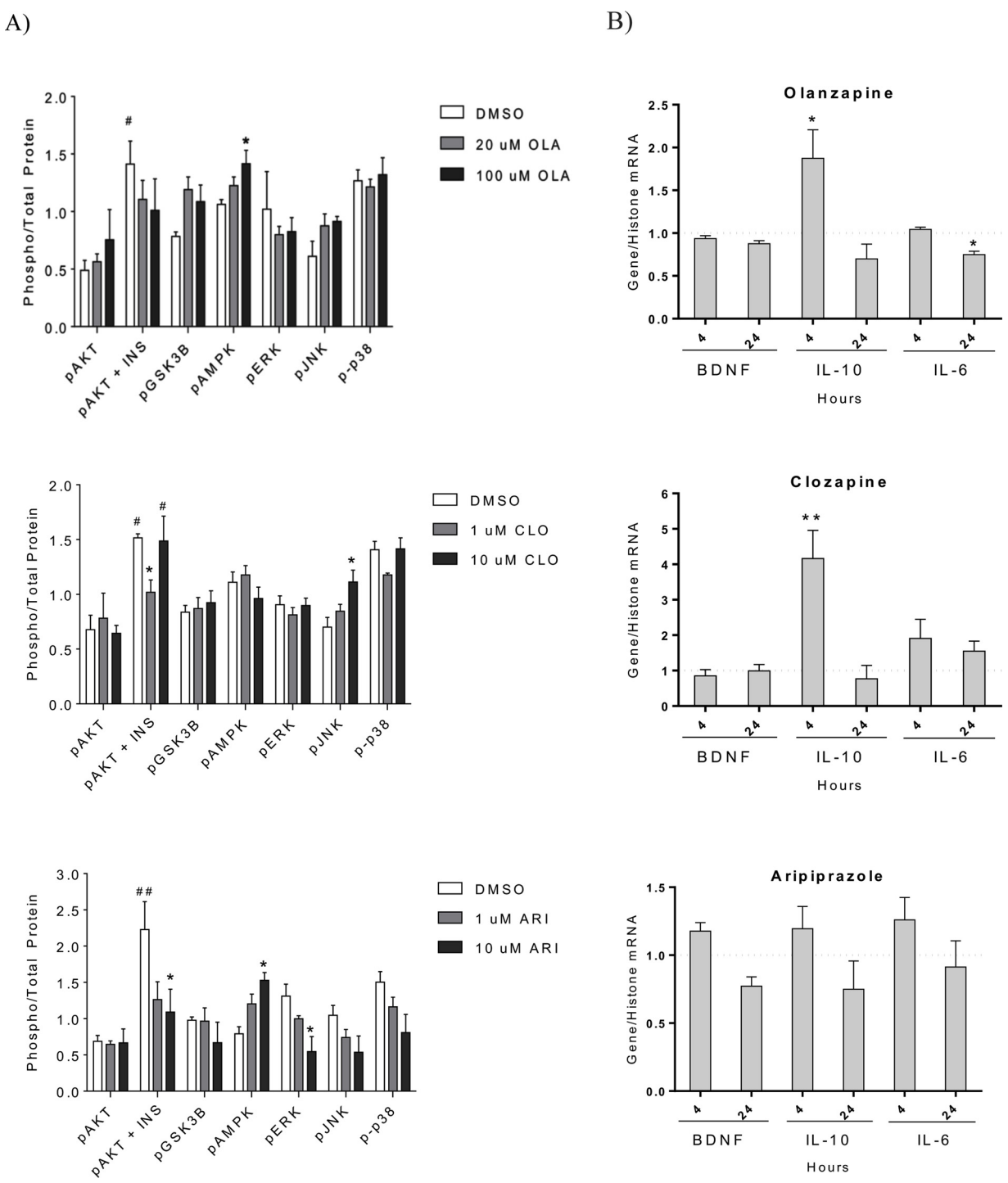

Fig. 1. OLA: olanzapine; CLO: clozapine; ARI: aripiprazole; INS: insulin. mHypoE-46 neurons were treated with OLA, CLO, or ARI. A) Western blot analysis was performed for proteins related to insulin signalling/energy sensing (AKT, GSK3B, AMPK), or MAPK proteins (ERK1/2, JNK, p38). Phospho-proteins were normalized to total proteins. *refers to effect of antipsychotic vs. DMSO: * $p < 0.05$; # refers to effect of INS stimulation relative to no stimulation (across treatment groups): #$p < 0.05$; ## $p < 0.01$; n = 4-5. B) RT-qPCR was used to measure mRNA expression of BDNF, IL-10, and IL-6. Vehicles were normalized to one and are represented by the dashed line (mean ± SEM.*$p < 0.05$; **$p < 0.01$; n = 3–5).

cell lines, a MTT assay was performed to assess cell viability in both the rHypoE-19 and rHypoE-46 cells. None of the APs (*i.e.*olanzapine (100 & 20 μM), clozapine (20 & 10 μM), and aripiprazole (20 & 10 μM) induced cell toxicity in either cell line over 24 h of incubation (Supplementary Fig. 1).

3.4. Cell line specific expression of neurotransmitter receptors

APs antagonize a variety of neurotransmitter receptors. The D_2 receptor is considered both necessary and sufficient for the clinical therapeutic effect, and all existing APs possess some degree of D_2 antagonism. Antagonism of the serotonergic class of receptors, primarily the $5HT_{2A/C}$ receptors, was initially thought to account for the so-called “atypicality” of the second-generation APs (historically defined

Table 1
OLA: olanzapine; CLO: clozapine; ARI: aripiprazole; AP: antipsychotic: INS: insulin. Summary of the effects of OLA, CLO, and ARI on protein and mRNA expression levels in mHypoE-46 neurons. Effects are indicated for AP versus DMSO, except for pAKT. For pAKT, analysis is run comparing the AP to the DMSO, as well as comparing the AP + INS to INS alone. The concentrations in uM are listed in brackets.

	Protein/Gene of Interest		OLA	CLO	ARI
Protein	pAKT*	AP + INS vs. INS	–	–	↓ (10)
		AP + INS vs. AP	–	↓(1)	↓ (10)
	pGSK3B		–	–	–
	pAMPK		↑ (100)	–	↑ (10)
	pERK1/2		–	–	↓ (10)
	pJNK		–	↑ (10)	
	p-p38		–	–	–
mRNA	BDNF		–	–	–
	IL-6		↓ (20)		–
	IL-10		↑ (20)	↑ (20)	–

by presence of an antipsychotic effect in absence of motor-related side effects). However, antagonism of the serotonergic receptors, in addition to the propensity of many of the higher metabolic liability APs to antagonize the H_1 and muscarinic-3 (M_3) receptors, has been associated with the clinical metabolic risk of these agents (Mauri et al., 2014). Thus, RT-qPCR was used to screen both the rHypoE-19 (studied in our previous work in the context of AP induced disturbances in energy and insulin sensing (Kowalchuk et al., 2019)), and mHypoE-46 neuronal lines for mRNA expression of the following receptors: the H_1, D_2, M_3, $5HT_{2A}$, and $5HT_{2C}$. The purpose of receptor screening was to explain or reconcile differences between the cell lines and explore potential mechanisms. The rHypoE-19 neurons expressed the H_1, $5HT_{2A}$, and M_3 receptors, with no detectable expression of D_2 or $5HT_{2C}$ receptors. Conversely, the mHypoE-46 neurons expressed the $5HT_{2A}$, D_2, and M_3 receptors, with no significant expression of the H_1 or $5HT_{2C}$ receptors.

4. Discussion

This study was undertaken in an attempt to replicate findings published by our group demonstrating effects across different APs on energy sensing and inflammatory pathways in a rat hypothalamic cell line (rHypoE-19) (Kowalchuk et al., 2019). Interestingly, in both the mHypoE-46 and rHypoE-19 neurons, clozapine and aripiprazole impaired insulin-induced pAKT increases, providing evidence of a potential metabolic impairment by antipsychotics *via*the hypothalamic insulin signalling pathway. There were also overlapping findings in energy sensing pathways. In the mHypoE-46 neurons, olanzapine and aripiprazole increased activation of the orexigenic protein AMPK, similar to the effects of clozapine and aripiprazole in the rHypoE-19 neurons. Previous *in vivo* work has linked increased AP-induced AMPK to antagonism of the H_1 receptor (He et al., 2014). In the rHypoE-19 cells, clozapine-induced pAMPK could be in agreement with this mechanism as the rHypoE-19 neurons express the H_1 receptor. However, olanzapine increased pAMPK in the mHypoE-46 neurons, which lack the H_1 receptor, suggesting that the H_1 receptor may not be required for AP-induced AMPK. In addition, we reported increased pAMPK with aripiprazole in both cell lines, which also does not support the involvement of the H_1 receptor as aripiprazole does not have prominent H_1 receptor antagonism. However, aripiprazole has previously been shown to increase pAMPK in non-neuronal cells through an H_1 independent mechanism (Takami et al., 2010). There is also a possibility that upregulation of pAMPK may involve the $5HT_{2A}$ receptor expressed in both lines, as there is some evidence that serotonin can regulate central AMPK Jeong et al., 2015.

Although we draw some similarities between the rat and mouse findings, the antipsychotics studied have variable effects on the two cell lines. For example, the evidence towards a pro-inflammatory effect of antipsychotics in the rHypoE-19 neurons (*via*upregulation of the MAPKs and IL-6) was not observed in the mouse cell line. Instead, we see a consistent anti-inflammatory effect: olanzapine decreased pro-inflammatory *IL-6* and increased anti-inflammatory *IL-10* mRNA expression, as well as a 4-fold increase in *IL-10* mRNA expression by clozapine. We also do not find the consistent induction of pJNK by all three antipsychotics that we saw with the rHypoE-19; instead, we see increased pJNK with clozapine (10 u M) alone. We do not believe the trend towards a pro-inflammatory profile in the rat neurons was due to drug toxicity, as the pro-inflammatory effects varied across antipsychotics (for example, clozapine had no effect on the cytokines in the rat neurons, but induced pJNK). We additionally conducted MTT assays which confirmed that reduced cell viability is unlikely to explain the differences in inflammatory responses observed between the two cell lines.

The differences seen between cell lines could be due to a number of factors. Tolerable dose differed between cell lines; the rHypoE-19 neurons tolerated much higher concentrations of antipsychotics compared to the mHypoE-46 neurons. Of interest, unique responses to hormones and other compounds can occur in what appear to be related cell lines (Nazarians-Armavil et al., 2013). As well, differences in the expression of receptors, including the receptors assessed in this study, may explain some of the variation between cell types. The species difference could also contribute to differences in the antipsychotic effects between cell lines, as inherent differences have been reported in the metabolic profiles of rats and mice (Menahan and Sobocinski, 1983). Moreover, differences in metabolic responses have also been reported within species; for example diet-induced obese C57BL/6J mice are more glucose intolerant, while diet-induced obese AKR/J mice are insulin resistant (Rossmeisl et al., 2003), along with strain differences in olanzapine-induced weight gain (Morgan et al., 2014)

A limitation of *in vitro* work is the uncertain clinical relevance of the chosen AP concentrations in relation to humans. In general, the concentrations we used are expected to be higher than known plasma AP concentrations. However, APs can accumulate in brain tissue at concentrations 10- to 30-fold higher compared to plasma (Aravagiri et al., 1999), and the concentrations used in this study correspond with those used in other *in vitro* work (Ardizzone et al., 2001; Takami et al., 2010).

In conclusion, olanzapine, clozapine, and aripiprazole all appear to have a direct effect on signal transduction pathways linked to metabolic function in the mHypoE-46 hypothalamic neurons. Within the consideration of differences in AP doses, species, and neurotransmitter receptor expression profiles, we replicate some similar findings in the present mouse hypothalamic cell line as compared to our previous findings in a rat cell line, including direct AP effects on pAKT and pAMPK. We also present novel findings suggesting that AP-induced upregulation of pAMPK (which has been linked to glucose dysregulation in *in vivo*rodent models) can occur *via*mechanisms independent of H_1 receptor antagonism. This may also help to explain the diabetogenic propensity of agents like aripiprazole. Further investigation of the mechanisms underlying these effects, and expansion of this work across other AP agents, and into *in vivo* rodent models, will provide insight into the differential action of these commonly prescribed medications in relation to whole body glucose homeostasis. Use of *in vitro* models to elucidate molecular pathways impacted by APs may in turn lead to studies examining targeted therapeutics to specific neuronal subpopulations in order to mitigate the detrimental metabolic side effects that limit tolerability of these agents.

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Declaration of Competing Interest

MH received consultant fees from Alkermes. All other authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.104400.

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