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The NOS1 variant rs6490121 is associated with variation in prefrontal function and gray matter density in healthy individuals.

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Abstract

A common polymorphism within the nitric oxide sythanse-1 (NOS1) gene (rs6490121), initially identified as risk variant for schizophrenia, has been associated with variation in working memory and IQ. Here we investigated how this variation might be mediated at the level of brain structure and function. In healthy individuals (N=157), voxel based morphometry was used to compare gray matter (GM) volume between homozygous and heterozygous carriers of the 'G' allele (i.e. the allele associated with impaired cognition and schizophrenia risk) and homozygous carriers of the non-risk 'A' allele. Functional brain imaging data were also acquired from 48 participants during performance of a spatial working memory (SWM) task, and analysed to determine any effect of NOS1 risk status. An *a priori* region-of-interest analysis identified a significant reduction in ventromedial prefrontal GM volume in 'G' allele carriers. Risk carriers also exhibited altered patterns of activation in the prefrontal cortex, caudate, and superior parietal lobe, which were characteristic of abnormal increases in activation in frontoparietal working memory networks *and* a failure to disengage regions of the default mode network. These functional changes suggest a NOS1-mediated processing inefficiency that may contribute to cognitive dysfunction in schizophrenia. While the mechanisms by which NOS1 may influence brain structure and/or function have not yet been well delineated, these data provide further evidence for a role of NOS1 in risk for schizophrenia via an impact upon cognitive function.

Keywords: nitric oxide synthase-1; schizophrenia; VBM; fMRI; medial prefrontal cortex.

1. Introduction

Neuronal nitric oxide synthase (nNOS) accounts for 90% of nitric oxide (NO) in the central nervous system, production of which is dynamically controlled during development and in response to brain injury (Khaldi et al., 2002). NO stimulates synthesis of cGMP, strongly influences glutamate neurotransmission (Akyol et al., 2004; Brenman and Bredt, 1997), and is involved in uptake, release and storage of other CNS neurotransmitters including acetylcholine, dopamine, noradrenaline, and GABA (Boehning and Snyder, 2003; Pepicelli et al., 2004). The NO synthase–1 gene (NOS1; OMIM 163731), which maps to chromosome 12q24 and encodes for nNOS, has been associated with several psychiatric illness phenotypes, including anxiety, depression, and schizophrenia (SZ) (Luciano et al., 2010; O'Donovan et al., 2008; Reif et al., 2009; Reif et al., 2010), yet evidence that NOS1 can explain illness risk is mixed. For example, following four positive NOS1 candidate gene associations in five studies (DeLisi et al., 2002; Fallin et al., 2005; Liou et al., 2003; Shinkai et al., 2002; Tang et al., 2008), a NOS1 single-nucleotide polymorphism (SNP) rs6490121 (located in intron 10; A/G polymorphism; minor allele is G) was identified by O'Donovan et al (2008) in a GWAS study as initially showing the strongest statistical evidence of association (p=9.82x10−6; risk allele 'G'). However this association was not replicated in subsequent SZ samples either in the O'Donovan et al study or other GWAS studies (Stefansson et al., 2009).

In contrast, NOS1 is robustly associated with cognitive variation. NOS1 mouse knockout models have repeatedly been associated with variance in cognition (Kirchner et al., 2004; Weitzdoerfer et al., 2004). Notably, phencyclidine hydrochloride–induced cognitive deficits that model SZ symptoms (e.g. pre-pulse inhibition, habituation of acoustic startle, latent inhibition, and deficits in spatial learning and memory and working memory) are

prevented by interfering with NO production (Johansson et al., 1997; Johansson et al., 1998; Klamer et al., 2001, 2004a, 2005; Klamer et al., 2004b; Palsson et al., 2007; Wass et al., 2006). In humans, Reif et al. (2006) reported that 2 of 4 genetic markers tested at the NOS1 locus were associated with variance in performance on measures of prefrontal function (i.e. 'Go/No-Go' paradigm). Similarly, two more recent investigations by the same group have found associations between NOS1 polymorphisms, cognitive performance and prefrontal brain function in patient with SZ (Reif et al., 2011) and healthy controls (Kopf et al., 2011). In light of these findings and our own observation that the putative risk 'G' allele identified by O'Donovan and colleagues at rs6490121 was associated with poorer performance in measures of verbal intelligence and working memory in SZ patients *and* healthy controls (Donohoe et al., 2009), we conclude that NOS1's more modest association with psychiatric risk may reflect the moderating effects of this gene's broader role in cognition.

With the aim of increasing our understanding of the mechanisms by which putative SZ risk variants in NOS1 may contribute to disease risk, this study considered the role of NOS1 rs6490121 in brain structure and function. We provide evidence that the 'G' allele, previously associated with lower verbal IQ and working memory, is associated with decreased grey matter (GM) volumes and an altered pattern of activation in complementary prefrontal cortical regions in healthy controls.

2. Materials and methods

2.1 Participants

2.1.1 Functional Imaging (fMRI): Healthy Irish (i.e. Irish born paternal and maternal grandparents), right-handed individuals (N=57) were recruited from the general population

through local media advertising. Participants provided written, informed consent, in accordance with local ethics committee guidelines. Due to data quality issues (e.g. head motion) and matching between genotype groups, 9 individuals were excluded. The remaining 48 individuals were all included in fMRI analysis (15 male; mean age=27.37 years; Table 1). Exclusion criteria included relevant neurological, medical or psychiatric history, family history of psychosis (i.e. one or more first degree-relative with a confirmed diagnosis of SZ or other psychosis), claustrophobia, pregnancy, and any other contraindication for MRI. Psychiatric history was confirmed prior to scanning using a semi-structured interview, in which participants responded to questions regarding whether or not they had ever seen a medical professional for a mental health issue or if they had ever been prescribed a psychiatric medication.

2.1.2 Structural Imaging (sMRI): Individuals were selected from the Trinity College Institute for Neuroscience imaging biobank project, which involved the opportunistic sampling of all healthy, right-handed controls participating in MRI studies at the Institute. Participants gave consent to the use of structural data collected under the primary study and provided a saliva sample for genetics analysis. From this sample, 157 individuals (71 male; mean age=27.71 years) were included based upon the quality of T1 structural data and successful genotyping of the NOS1 (rs6490121) variant. Forty six sMRI participants were also included in fMRI analyses.

The majority of participants (N=120) were confirmed to be Irish. Lineage information was not available for remaining participants (N=37). However, given the relative homogeneity of the Irish population (e.g. 92% of Dublin city population are Irish/Caucasian; www.cso.ie/census), the vast majority of these participants were also likely to have been of Irish lineage. Furthermore, this subgroup did not differ from known Irish participants in terms of genotype frequency, gender, age, or VBM analysis of GM/white matter (WM) volume.

>> Table 1<<

2.2 Procedure

2.2.1 Genotyping: Genetics analysis was carried out using DNA obtained from saliva samples that were collected using Oragene DNA self-collection kits (DNA Genotek). The rs6490121 SNP was genotyped using a Taqman® SNP Genotyping Assay on a 7900HT Sequence Detection System (Applied Biosystems). The call rate for the Taqman genotyping was >95% and the samples were in Hardy-Weinberg Equilibrium ($p>0.05$). In addition a small number of HapMap CEU DNA samples (www.hapmap.org) were genotyped for rs6490121 for quality control purposes and were all found to be concordant with available online HapMap data for this SNP. Genotype frequencies for fMRI and sMRI are noted in Table 1.

2.2.2 MRI: Participants were imaged on a Philips Intera Achieva 3T MR system. Whole-brain BOLD EPI consisting of 32 non-contiguous, axial 3.5mm slices was acquired with a 0.35mm slice gap and the following imaging parameters: TR=2000ms; TE=35ms; FOV=224x224mm at 64x64 matrix; and flip angle=90°. The duration of functional scanning was 220TRs/440s. Structural imaging involved the acquisition of a T1-weighted image (180 slices; duration=6mins) using a TFE gradient echo pulse sequence, with a slice thickness of 0.9mm and 230x230mm FOV.

2.2.3 SWM paradigm (Figure 1): In this block-design task participants were asked to determine whether the spatial location, relative to a white fixation cross, of a white dot (i.e. target) and a red circle (i.e. probe) were either the same (*match*) or different (*no match*). The target and probe were each presented for 500ms. There were three levels/conditions in the task: A. No Delay (baseline); B. 1-dot ; and C. 3-dot. In the 'no delay' condition the target was a single white dot, and both the target and probe appeared simultaneously (Figure 1A).

Conversely, during the 1-dot condition (Figure 1B) the target and probe were separated by a 3sec delay. Similarly, the 3-dot condition (Figure 1C) also incorporated a 3sec inter-stimulus interval, however in this condition the target image was comprised of 3 dots. During the 3-dot condition the probe was also a single red circle and participants were asked to judge whether the probe was in the same position as *any one* of the three dots in the image that preceded it.

There were 6 trials per block with an inter-trial interval of 2sec. Participants completed 4 blocks of each condition (i.e. 12 blocks/72 trials total). The order of blocks was fixed, however to account for the potentially confounding effect of block order on brain activity across the session, there were two predetermined block orders and participants were pseudo-randomly allocated to one or the other prior to imaging.

Participants were given left- and right-hand MRI compatible response units. They responded with a left button press for a 'match' and a right button press for 'no match'. While there were equal numbers of 'match' and 'no match' trials, 20% trials were defined as 'difficult'; whereby the probe was in a similar (i.e. same quadrant) but different location to the target. The number of difficult trials was consistent across task levels and between participants. The definition of trials as 'difficult' was relative to other trials, and was not based upon participant's perceived difficulty of these trials.

Behavioural measures of interest on the SWM task were accuracy (i.e. number of correctly identified trials) and reaction time (RT).

>>Figure 1<<

2.3 Data Analysis

2.3.1 fMRI: Functional imaging data were analyzed using Analysis of Functional Images (AFNI; Cox, 1996). To correct for head motion, 3D EPI data for each subject were co-registered to a base volume. The subsequent data were inspected for motion using the censor.py application for AFNI (http://brainimaging.waisman.wisc.edu/~perlman/code/censor.py), using the following censoring criteria: translation>0.3mm or rotation>0.3° between consecutive TRs. TRs exceeding these parameters were recorded and were removed from further analysis prior to deconvolution. Individuals with a censor rate >25% of TRs were excluded from analysis (i.e. N=2). For the remaining participants (i.e. N=48) the mean percentage of TRs censored was 3.29% (range: 0-21.82), which was equivalent to 7.25TRs (range: 0-48). Almost 30% of participants required no censoring (i.e. 14 of 48), while 67% had less than 25 TRs censored. The remaining subjects had 42 and 48 TRs censored each. Visual inspection of censor plots indicated that those TRs that were censored were relatively evenly distributed across the session and block types. Thus, we do not anticipate any significant effect of censoring on the ability to accurately construct task regressors for those subjects who were included in fMRI analysis. Data quality control also included the application of an edge detection algorithm to exclude activations occurring outside the brain.

Voxel-wise multiple regression was conducted in which imaging regressors were expressed as a delta function relative to baseline and convolved with a hemodynamic response function. There were regressors representing the SWM conditions (1-dot and 3-dot) and six motion parameters that were included as regressors of no interest. A voxel-wise average amplitude change (β) equal to the percentage change from baseline was calculated for each SWM condition. The resultant activation maps for each subject were the registered to a higher resolution ($1\mu l/mm^3$) standard space (Talairach and Tournoux, 1988) and spatially blurred using a 4.1mm Gaussian isotropic kernel.

Whole-brain mixed measures ANOVA were used to consider the impact of genotype group and SWM condition (1-dot vs. 3-dot) on brain activity. In light of the relatively small number of G homozygotes (N=7), imaging analyses focussed on the relative differences in non-risk homozygous (i.e. AA) vs. risk carrying (i.e. AG & GG) individuals. These groups were matched for age, gender, and years of education (Table 1).

A voxel-wise threshold correcting for multiple comparisons and controlling for family-wise error (FWE) rate was calculated using a Monte Carlo simulation. This deterministic sampling algorithm ascertains the frequency of significant clusters that would occur by chance under the null hypothesis (i.e. the false positive rate). Cluster significance was determined as equalling or exceeding a given extent threshold at $p_{CORRECTED}<0.05$ following corrections for multiple comparisons at the whole brain level

Given previous findings suggesting an impact of the NOS1 genotype on mPFC and cognitive processes that are mediated by function in prefrontal (PFC) and parietal cortices, such as executive function, *a priori* region-of-interest (ROI) analyses were also carried out. These analyses focussed on the impact of genotype on brain activity in medial PFC (mPFC)/BA10, dorsolateral PFC (dlPFC)/BA9 & 46 and superior parietal cortex/BA7. These regions were defined using a Talairach template in AFNI. The mean value across voxels within each ROI was calculated for each participant/regressor and subsequently used as the dependent variable in statistical analyses.

2.3.2 Voxel based morphometry (VBM; Ashburner and Friston, 2000): sMRI analysis was performed within SPM5 (http://www.fil.ion.ucl.ac.uk/spm) running under Matlab (v7.8; The MathWorks) and utilising the VBM toolbox (v5.1; http://dbm.neuro.uni-hen.de/vbm). Individual volumes were visually inspected for scanner artefacts and gross anatomical abnormalities. Volumes that passed initial data quality control, were segmented into GM,

WM and cerebrospinal fluid, without tissue priors and using a Hidden Markov Random Field weighting of 0.15. Segmented images were normalised using the DARTEL toolbox (Ashburner, 2007), in which GM and WM templates were created using standard parameters. Jacobian scaled ('modulated') warped tissue classes were subsequently created for both GM and WM for each subject. The resultant images were smoothed with an $8mm^3$ Gaussian kernel.

The statistical methods employed for sMRI analyses were specifically designed to mimic those carried out for fMRI data. Mixed model ANOVA considering the impact of NOS1 genotype (AA vs. AG/GG) on GM and WM density were conducted for both whole brain and *a priori* ROI. The same regions were included as for the functional ROI analyses, and were defined using the WFU pickatlas toolbox for SPM (Maldjian et al 2003). Although the groups were essentially matched for age, gender, and years of education (Table 1), to account for normal variation in brain structure, age, gender and total GM or WM volume were included as co-variates. For these analyses, we included subject data as normalised to native space (i.e. the DARTEL template) rather than a standard template, such as MNI. Subjects for sMRI were sampled from a range of imaging studies that included individuals in a range of developmental stage, including young, middle, and late adulthood, making normalisation to a standard template inappropriate. As with fMRI analyses, significance was defined as the voxel-wise threshold at FWE $p_{CORRECTED}<0.05$ for whole-brain analyses or for the entire volume of the mask in the case of ROI analyses. Since non-uniform smoothness of VBM data can influence interpretation of these types of analysis (Ashburner and Friston, 2000; Worsley et al., 1999), determination of significance included a non-stationarity cluster extent correction, which utilized the random field theory version of cluster inference under non-stationarity (Hayasaka et al., 2004) and was implemented using the NS toolbox (http://fmri.wfubmc.edu/cms/NS-General).

2.3.3 Other data: Behavioural and demographic data were analysed in SPSS (v16; SPSS Inc.).

3. Results

3. 1 SWM behavioural data

There was a significant main effect of SWM condition on participant accuracy ($F_{(2, 90)}$=22.51, p<0.001). Although the average number of correct responses did not differ between the no-delay and 1-dot conditions, participants made significantly more errors on the 3-dot condition compared to the no-delay ($t_{(46)}$=-5.34, p<0.001) *and* the 1-dot condition ($t_{(46)}$=- 6.77, p<0.001). In addition, there was a linear effect of task difficulty on reaction time (RT; $F_{(2,90)}$=18.19, p<0.001), such that as the task got more difficult RT increased. There were no effects of NOS1 genotype on RT or accuracy, and no interactions between genotype and SWM condition on either behavioural measure.

One potential issue here is that, due to the relatively small number of participants, our study may have been underpowered to detect behavioural differences between the genotype groups. Estimates of effect size (i.e. partial η^2) indicated that any variability in accuracy that could be accounted for by genotype was negligible (i.e. genotype η^2=0.001; genotype x SWM η^2=0.005). Conversely, for RT estimates of effect size (i.e. genotype η^2=0.02; genotype x SWM η^2=0.04) suggest that there may be some small contribution of NOS1 genotype to variability in RT on measures of executive function. Overall, these data suggest that even at much larger sample sizes it is unlikely that there would be any effect of genotype on accuracy on this SWM measure, but that it might be possible to observe a small but significant effect of genotype on RT.

3.2 fMRI

3.2.1 Main effect of SWM maintenance on brain activity (Table 2): SWM condition influenced brain activity bilaterally in two large clusters centred in the middle temporal gyrus and which extended to occipital and parietal cortices, in the left angular gyrus/BA39, posterior cingulate/BA31, inferior frontal gyrus/BA9 and middle frontal gyrus/BA8, in the right culmen and the bilateral precentral gyrus/BA6. In the two primary clusters and in BA6 and BA9 this effect was result of a significantly greater increase in activation in response to the 3-dot, vs. 1-dot, condition. Conversely, activity was lower in response to 3-dot trials in posterior cingulate, middle frontal gyrus, and BA39. These latter regions all showed comparatively reduced (i.e. relative to baseline) activation on 1-dot trials; an effect that was even more pronounced for 3-dot trials. This pattern of activity is in keeping with theories of frontoparietal activation and corresponding deactivation in regions constituting the default mode network during executive function (Fox et al 2005; Toro et al 2008). Thus, we were assured of the robustness of the SWM task.

>> Table 2<<

3.2.2 Main effect of NOS1 genotype (Figure 2; Table 3): Irrespective of SWM condition, NOS1 genotype impacted upon brain activity in the right caudate, superior parietal lobe, superior frontal gyrus and cuneus/BA7 ($p_{CORRECTED}<0.05$). In all significant clusters, activation was *greater* in individuals who were carriers of the NOS1 risk 'G' allele (*post hoc* t test: $p<0.001$).

>> Figure 2<<

3.2.3 Interaction between NOS1 genotype and SWM condition (Figure 3; Table 3): There was an interaction between genotype and condition in the left lingual and middle

frontal gyri. In the lingual gyrus activity in the 1-dot condition did not differ between groups, however, activation in risk 'G' allele carriers was greater in response to 3-dot trials ($p<0.05$). Moreover, risk carriers showed a main effect of working memory load (1-dot < 3-dot; $p<0.001$) that was absent in the non-risk homozygotes. In the middle frontal gyrus, non-risk individuals exhibited a notable decrease in activity in response to 3-dot, but not 1-dot trials ($p<0.001$), while opposite was true in risk carriers (i.e. 1-dot > 3-dot; $p<0.001$). In addition the extent of deactivation in response to 3-dot trials was greater in non-risk homozygotes vs. risk carrying individuals ($p<0.05$).

Post hoc one-sample t-tests confirmed that the extent of deactivation for risk carriers on 3-dot trials and non-risk individuals on 1-dot trials did not differ significantly from 0 ($p>0.05$).

3.2.4 A priori ROI results: Within those regions that were included in the region-of-interest analysis there was a main effect of SWM difficulty in dlPFC (i.e. BA46; 1-dot < 3-dot; $p<0.05$). There was also a main effect of genotype in BA10 and BA7, such that risk carriers showed relatively greater activation compared to non-risk individuals in both regions, irrespective of load ($p<0.05$).

>> Figure 3<<

>>Table 3<<

3.3 VBM

3.3.1 NOS1 genotype and GM (Figure 3): There were no regions of significantly different GM density between non-risk individuals and risk carriers that survived correction (FWE) for multiple comparisons at the whole-brain level.

The *a priori* ROI analysis, on the other hand, was indicative of a NOS1-mediated influence on GM density in mPFC/ BA10, such that GM volume was relatively reduced in risk carriers, vs. non-risk individuals, in a single cluster in the more lateral aspects of ventromedial PFC (vmPFC; MNI co-ordinates: 19 61 26; K_E=371 voxels; $p_{CORRECTED}$<0.05; Figure 4). There were no between-group differences in GM density in dlPFC or parietal cortex.

>>Figure 4<<

3.3.2 NOS1 genotype and WM: Following correction for multiple comparisons, there were no regions of WM that were found to be sensitive to NOS1/rs6490121 genotype.

4. Discussion

In this investigation VBM and fMRI were used to determine the impact of the NOS1 variant rs6490121 on brain structure and function in healthy controls. Carriers of the SZ risk 'G' allele had comparatively reduced GM volume in lateral vmPFC, compared to individuals who were homozygous for the non-risk 'A' allele. Similarly, risk carriers showed a pattern of activation during performance of a SWM task which differentiated them from the non-risk individuals. In a select range of areas in prefrontal and parietal cortices, including BA10/vmPFC, risk carriers exhibited a load-independent increase in activity compared to homozygous non-risk individuals. Furthermore, carriers of the risk 'G' allele also exhibited load-dependent changes in activity in the lingual and middle frontal gyri, such that they failed to disengage BA8 with increasing memory load and inappropriately increased activity in the lingual gyrus as the task got more difficult.

4.1 Functional effects of NOS1

Whole brain and ROI analyses implicated NOS1/rs6490121 with load-independent increases in activation in the mPFC/BA10. This supports prior observations showing that activity related to cognitive tasks that involve mPFC are influenced by NOS1 genotype (Reif et al., 2006). Functionally, the mPFC is one of the regions implicated in the so-called 'default mode network' (i.e. those regions that are involved in self-referential mental activity and which show reduced activity during goal directed beahviour; Buckner et al., 2008; Gusnard et al., 2001; Raichle et al., 2001). Similarly, of those regions where we noted interactions between genotype and working memory load (i.e. lingual gyrus and middle frontal gyrus/BA8), BA8 has also been shown to exhibit goal-related decreases in activation (McKiernan et al. 2003). Risk-related variability in function also included increased activity in BA7 and the caudate. The superior parietal region is a key component of the frontoparietal network that supports executive function (Fox et al 2005), while the caudate is thought to play an integral role in the cortico-striatal-cortical connections which facilitate communication between frontal and parietal regions during working memory tasks (Joel and Weiner 2000). Collectively, these data suggest that those who were carriers of the risk 'G' allele attained a level of performance equivalent to their non-risk peers via a combination of over-activation in regions that are necessary for executive function and failing to disengage regions that would not normally be involved in goal-directed behaviour.

It has been suggested that impaired accuracy in measures of working memory are related to processing inefficiency in frontoparietal networks (Bassett et al. 2009), and that cognitive deficits in SZ may reflect 'dysconnection' of functional brain networks that are involved in optimal cognitive performance. Moreover, variability in SZ susceptibility genes may contribute to dysfunctional activity during cognitive processing in SZ (Tan et al 2007) via an impact on the co-ordination of functional networks in patients. Therefore, the

influence of NOS1 on brain activity associated with SWM seen here suggests that NOS1 may contribute to SZ risk via an impact on processing efficiency in aspects of frontoparietal and default mode networks.

Given the paucity of human research on NOS1, it is interesting to speculate as to the neural mechanism(s) by which the effects of the rs6490121 NOS1 variant seen here might arise. With regards to how NOS1 may specifically impact upon brain function, there are a number of NO attributes that may contribute to fluctuations in the BOLD signal as measured by fMRI. For example, NO functions as a neurotransmitter (Garthwaite et al., 1988), interacts with other neurotransmitters (Boehning and Snyder, 2003; Pepicelli et al., 2004) and is involved in vasodilation and blood flow (Toda et al., 2009a, b). It is unlikely that vasodilatory effects contributed to between-group differences in activity seen here, since a global effect of NOS1/NO on blood flow (increase or decrease), rather than a region-specific change, would be more likely, and this would produce a signal that was additive to the effect of interest (Corfield et al., 2001). Rather, it seems more plausible that the impact of NOS1 genotype on brain activity is at the neurotransmitter level. For example, NO impacts upon the function of other neurotransmitters such as serotonin, glutamate and dopamine, which are functionally relevant for neuronal pathways that innervate some of the regions shown here to exhibit an effect of NOS1 genoytpe (e.g. dopamine in the caudate and mPFC; serotonin and glutamate in parietal cortices). NO increases GABA release (Getting et al., 1996; Ohkuma et al., 1996), stimulates the release of noradrenaline and glutamate (Lonart et al., 1992) and increases the release of dopamine and serotonin (Kaehler et al., 1999; Lorrain and Hull, 1993). Presuming a positive association between NO and the release of these neurotransmitters, enhanced activity in prefrontal and parietal cortices seen here be associated with an excess of NO in carriers of the risk 'G' allele and subsequent overstimulation of brain regions innervated by pathways involving neurotransmitters whose levels are exaggerated.

4.2. Structural effects of NOS1

Our observations suggest that in addition to effects on brain activity NOS1 also impacts upon brain structure in vmPFC. Interestingly, a recent VBM investigation of the structural correlates of cognitive function in SZ found that reduced GM volume in mPFC, bilaterally, was associated with dysexecutive behaviour in patients (Kawada et al 2009). Thus, NOS1-mediated effects on GM volume may also contribute to abnormalities in working memory function. However, the mechanisms by which NOS1-related changes in brain structure occur may not necessarily overlap with those potentially impacting upon brain activity. As noted, NOS1 plays an important role in neurodevelopment and the response to brain injury via production of NO in the CNS (Khaldi et al., 2002). NO has the potential to reduce the extent of neuronal damage via mechanisms leading to a decrease in toxicity following reperfusion (Chiueh, 1994, 1999; Pluta et al., 2001) or reduced sensitivity to NMDA-mediated toxicity (Khaldi et al., 2002). Conversely, excessive amounts of NO can contribute to cell death via a variety of mechanisms (Brown, 2010) such as cellular damage (Calabrese et al., 2007) and apoptosis (Li and Wogan, 2005). Further, excessive NO is a common feature of neurodegenerative disorders (Calabrese et al., 2007) and although SZ is primarily considered to be a neurodevelopmental disorder (Weinberger, 1987), neuroimaging data in humans suggests that it is also associated with progressive neurodegeneration (Csernansky, 2007). While the contribution of NO to SZ remains to be fully elucidated (Bernstein et al., 2005), it is likely that downstream consequences of NOS1 variants that compromise either the neuroprotective role of NO and/or which contribute to neurodegeneration via the neurotoxic role of excess NO are key to this phenomenon.

4.3 Molecular mechanisms of NOS1

The implicated SNP (rs6490121), located in intron 10 of *NOS1*, has no obvious functional impact and may reflect a proxy association with another causal variant(s). As we noted previously (Donohoe et al., 2009), rs6490121 is not in high linkage disequilibrium (LD; r^2 >0.8) with any other common SNP at this locus based on HapMap CEU data. Previously reported data from our lab (Donohoe et al., 2009) indicates that the risk G allele of rs6490121 is in partial LD (D′=0.70, r^2=0.26, HapMap CEU) with the short alleles of a variable number tandem repeat (VNTR) located in the promoter regions of exon 1f (known as NOS1 Ex1f VNTR; Hall et al., 1994). This VNTR is associated with decreased transcriptional activity of the NOS1 exon 1f promoter, alterations in the neuronal transcriptome impacting other putative SZ susceptibility genes (RGS4 and GRIN1), and affects both electrocortical and neuroimaging measures of PFC functions underlying cognitive control (Reif et al., 2009; Kopf et al., 2011a; Kopf et al., 2011b). While this suggests a possible functional mechanism of the snp reported on here, a previous study that directly investigated the effects of this VNTR in our samples revealed no association with any neuropsychological measure. Apart from Bunocore et al.'s (2010) allelic expression study of another NOS1 variant (rs1047735, which is not in LD with the variants reported here), we are unaware of NOS1 that have directly investigated the function of this variant. Relevant to NOS1's role in cognition, a recent study by Nicodemus et al. (2010) investigated epistasis between SZ-implicated genes lying within the NMDA receptor pathway, including NRGI, ERBB4, and AKT1. No evidence of epistasis was reported for the four NOS1 SNPs studied, although these did not include rs6490121. More systematic studies NOS1's involvement in protein expression and regulation and adaptor proteins linked to synaptic function and plasticity are clearly warranted, particularly in relation to the rs6490121 given

the accumulating evidence for its functional effects both here and previously (Donohoe et al, 2009; O'Donoghue et al, 2011).

4.4 Study Limitations

Inevitably, interpretation of these data is limited by a number of factors. First, due to the relatively small percentage of homozygous 'G' individuals, our study was underpowered to determine whether or not there is a dose effect of the risk 'G' alleles on measures of brain structure or function. In our earlier behavioural study we observed that the deleterious neuropsychological impact of rs6490121 was particularly noticeable in homozygous 'G' carriers in samples of more than 400 participants (Donohoe et al., 2009). An important issue for future imaging studies of this variant will be determining whether the effects observed here are particularly associated with homozygous 'G' carriers.

A further issue for our study, given the opportunistic manner by which the sMRI sample was collected, was the unknown genetic background of some participants. As noted, given the homogeneity of the Irish population, the vast majority of these cases were almost certainly of Irish or European lineage. Further, the minor allele frequency here did not diverge from that reported in our previous study and so is highly unlikely to have materially influenced the significance of our results.

There are also limitations related to fMRI experimental design. Firstly, although relatively large for a functional imaging study, our fMRI sample size was relatively small for a genetic study. Therefore, replication in a larger sample would be advantageous. A second issue related to experimental design is the fact that the potential influence of NOS1 in the brain is widespread, yet we saw regionally-specific effects of genotype during functional imaging. It is likely that this is simply a consequence of sensitivity to functional changes that was determined by the task that we used for functional activation (i.e. SWM) and that other

functional probes would reveal differences in additional or alternative regions. To clarify this future studies should explore whether the variation in this particular NOS1 SNP also influences other cognitive and affective processes and their neural correlates.

A third design-related factor is the lack of an effect of NOS1/rs6490121 on accuracy; a result which is in contrast to our previous findings (Donohoe et al., 2009). Effect size calculations indicated that this was not a power issue. These between-study differences may be related to distinct neural mechanisms underlying the spatial working memory task used here and the verbal task used previously. Alternatively, they may reflect the simplicity of the experimental paradigm. For example, despite an effect of load on accuracy, performance rates were high across task levels. This lack of overall variability in performance may have reduced our sensitivity to NOS1 effects on accuracy. Functional imaging studies employing spatial *and* verbal variants of more difficult working memory paradigms may be warranted to further delineate the potential influence of NOS1 on working memory-related function.

It also seems pertinent to note that the effects seen here were specific to healthy individuals, and exploration of the impact of this variant on brain function and structure in SZ patients would be advantageous in determining the role of NOS1 in the disease phenotype.

Finally, the functional role of the brain regions highlighted in the SWM analyses are complex and the interpretation offered here regarding the role of NOS1 on brain activity reflects our attempt to outline the most plausible explanation. Nonetheless, there may be other factors at play and in the absence of additional and/or replication data, caution should be exercised in the interpretation of the precise role of NOS1 on brain activity related to executive function.

4.5 Conclusions

In sum, the data presented here highlight the importance of NOS1 SNP rs6490121 in brain structure and function. The mechanisms by which this intronic SNP influences either of these factors and whether they are causally linked remains to be established, but may result from NO-mediated influences on neurodegeneration and neurotransmitter function. That effects of NOS1 were seen in regions of the brain involved in executive function and default activity in healthy individuals further supports the notion that this SNP contributes to SZ risk via an influence on goal-directed cognitive processes. Indeed, our data suggest that the influence of rs6490121 on SZ risk may be due to a pattern of cortical inefficiency that can be characterized by abnormal activity in frontoparietal networks that are involved in executive function, combined with a failure to disengage default mode regions during goal-directed behaviour.

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Tables

Table 1: Summary of participant demographics

	Total Sample	***AA***	***AG***	***GG***	***Comparison (AA vs. AG/GG)***
fMRI					
	N=48	N=24	N=17	N=7	
Gender (M:F)	15: 33	7: 17	6: 11	2: 5	ns
Age (mean s.d.))	27.37 (12.17)	28.21 (13.02)	22.12 (15.78)	37.29 (15.03)	ns
Years of Education (mean (s.d.))	17.36 (1.67)	17.24 (1.84)	17.47 (1.51)	17.50 (1.76)	ns
VBM					
	N=157	N=73	N=65	N=19	
Gender (M:F)	71: 86	32: 41	29: 36	10: 9	ns
Age (mean s.d.)	27.71 (12.98)	26.86 (12.79)	26.98 (11.84)	33.47 (16.35)	ns
Years of Education (mean (s.d.)	16.92 (2.30)	17.12 (2.41)	16.90 (2.23)	16.29 (2.17)	ns

Table 2: Main effect of SWM maintenance condition on brain activity. Note: all regions shown are significant at $p_{CORRECTED}<0.05$ (minimum cluster extent=775 voxels); **=p<0.001.

	Talairach co-ordinates					
Region	***x***	***y***	***z***	***Vol. (mm^3)***	***F***	***Post hoc t***
Right middle temporal gyrus	31	-69	18	62077	$F_{(1,46)}$=84.53**	*1-dot<3-dot***
Left middle temporal gyrus	-29	-72	19	37953	$F_{(1,46)}$=80.06**	*1-dot<3-dot***
Left posterior cingulate/BA31	-3	-59	23	16212	$F_{(1,46)}$=46.69**	*1-dot>3-dot***
Right middle temporal gyrus/BA39	51	-59	11	5258	$F_{(1,46)}$=39.66**	*1-dot>3-dot***
Left angular gyrus/BA39	-45	-72	30	3740	$F_{(1,46)}$=24.61**	*1-dot>3-dot***
Right precentral gyrus/BA6	27	-11	51	3420	$F_{(1,46)}$=51.26**	*1-dot<3-dot***
Left precentral gyrus/BA6	-25	-10	51	2730	$F_{(1,46)}$=52.04**	*1-dot<3-dot***
Left inferior frontal gyrus/BA9	-57	5	29	1889	$F_{(1,46)}$=34.58**	*1-dot<3-dot***
Right culmen	7	-53	-3	1234	$F_{(1,46)}$=19.96**	*1-dot>3-dot***
Left middle frontal gyrus/BA8	-25	31	46	789	$F_{(1,46)}$=20.22**	*1-dot>3-dot***

Table 3: The effect of NOS1/rs6490121 genotype on brain activity. Note: all regions shown are significant at $p_{CORRECTED}<0.05$; *post hoc* t-test *p<0.05; **=p<0.001.

	Talairach co-ordinates					
	x	***y***	***z***	***Vol. (mm^3)***	***F***	***Post hoc t***
Genotype						
Right caudate	11	16	-5	1155	52.03**	AA < AG/GG**
Right superior parietal lobe/BA7	24	-68	43	633	21.95**	AA < AG/GG**
Right superior frontal gyrus	17	55	8	545	25.01**	AA < AG/GG**
Right cuneus/BA7	6	-75	33	420	17.78**	AA < AG/GG**
Genotype x SWM						
Left lingual gyrus	-21	-90	-1	976	27.26**	3-dot: AA < AG/GG* AG/GG: 1-dot < 3dot**
Left middle frontal gyrus	-38	24	45	489	24.99**	3-dot: AA<AG/GG* AA: 1-dot > 3-dot** AG/GG: 1-dot<3-dot**

Figure Captions

Figure 1: Spatial working memory paradigm

Figure 2: The main effect of NOS1 (rs6490121) genotype (AA vs. AG/GG) on brain activity. **A**: Regions of significant (i.e. F>12.16; $p_{CORRECTED}$<0.05; minimum cluster extent=306 voxels/mm^3) activity associated with NOS1 genotype - i.e. caudate (vol.=1155mm^3), superior parietal lobe (vol.=633mm^3), superior frontal gyrus (vol.=545mm^3) and cuneus/BA7 (vol.=420mm^3). Clusters are shown on the ICBM452 T1 template from AFNI (Cox, 1996). **B**: Mean signal change (β) in each of these significant clusters (co-ordinates formatted as RAS) during 1-dot and 3-dot conditions in each participant group. Note: ***=p<0.001; **=p<0.005.

Figure 3: Regions demonstrating a significant (i.e. F>12.16; $p_{CORRECTED}$<0.05; minimum cluster extent>306 voxels/mm^3) interaction between NOS1 (rs6490121) genotype (AA vs. AG/GG) and SWM maintenance condition (1-dot vs. 3-dot). **A**. Left lingual gyrus (vol.=976mm^3). **B**. Left middle frontal gyrus (vol.=489mm^3) Note: Clusters are rendered on the ICBM452 T1 template from AFNI (Cox, 1996). *=p<0.05.

Figure 4: Regions of gray matter exhibiting an effect of NOS1 (rs6490121) on brain volume in healthy controls in *a priori* ROI analysis – mPFC/BA10; MNI co-ordinates: 19 61 26; K_E=371 voxels; cluster level $p_{CORRECTED}$<0.05. Note: Clusters are rendered on the ch2better brain template from MRIcron (www.nitrc.org/projects/mricron)

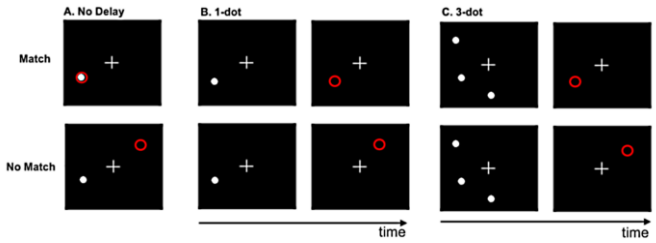

Fig. 1

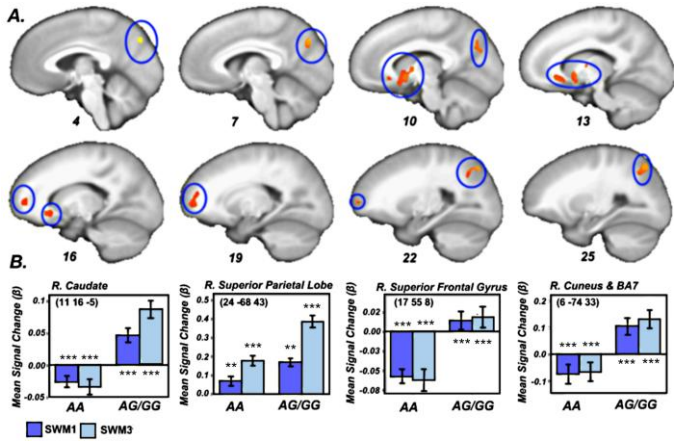

Fig. 2

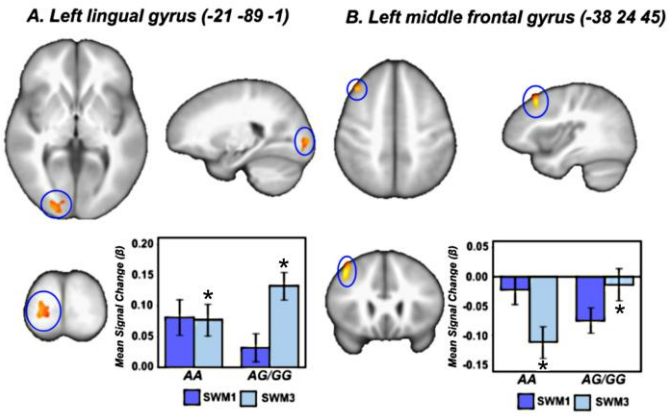

Fig. 3

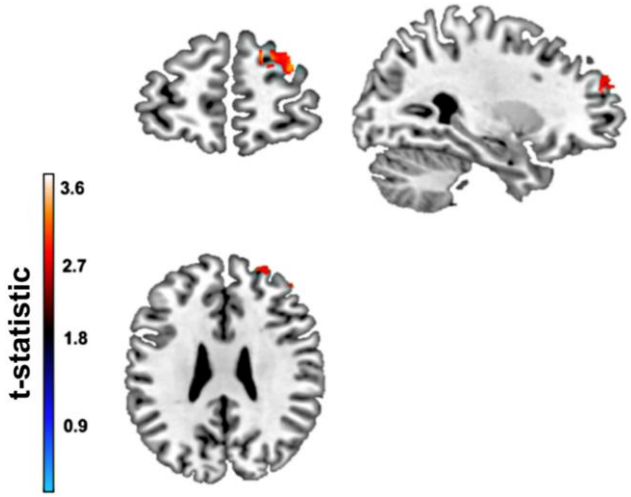

Fig. 4