Supplementary material for

Sex matters – a multivariate pattern analysis of sex- and gender-related neuroanatomical differences in cis- and transgender individuals using structural magnetic resonance imaging

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Sexual Orientation

An important factor needing consideration, when investigating sex- and gender differences, is sexual orientation, referring to the physical and emotional attraction to other persons of the same and/or opposite biological sex. Several studies showed that sexual preference has neurobiological correlates in both measures of cerebral anatomy and function (Byne and others 2001; LeVay 1991; Manzouri and Savic 2018a; Ponseti and others 2007). However, the available findings, mostly based on single brain structures, are limited in terms of comparability and do lack replication. Overall, the majority of the reported sex-, gender-, and sexual orientation-related differences in brain structure are still inconclusive, which may be due to the univariate statistical approaches' weakness in modelling complex but salient dimorphic patterns underlying sex and gender-related brain variation.

Here, sexual orientation of study participants was determined using a modified version of the Klein Sexual Orientation Grid (Klein et al., 1985) at screening visit. In order to unify the notion of sexual orientation, here, we used the item "Sexual Attraction" assessed on a 7-point scale with 1 = "attraction to women only" and 7 = "attraction to men only" at present and computed sexual orientation based on biological sex (1-2 attracted to women, 3-5 bisexual, 6-7 attracted to men). Of note, our classification must not necessarily match with the self-reported sexual orientation of our participants as the latter might rather be based on gender identity. Missing data (3 missing values) were imputed using a seven nearest neighbor approach based on the Euclidian distance (Troyanskaya et al., 2001). For instance, a biological man reporting a sexual attraction towards women was classified as "heterosexual" in our analysis irrespective of the gender identity (male or female).

The distribution of sexual orientation calculated by means of a χ^2 test was significantly different across groups in our sample (χ^2=34.69, p<0.001, see Table 1). To estimate the potential influence of sexual orientation on our MVPA findings, a two-way ANOVA was

calculated using the decision scores generated by the models that were proven significant as dependent variable and sexual orientation (hetero-, bi- and homosexual) and sex as fixed factors. Two-way ANOVA revealed a main effect of sex ($F=39.8$, $p<0.001$) but not sexual orientation ($F=0.7$, $p=0.52$) and no interaction effect ($F=2.3$, $p=0.12$). Similarly, there was a main effect of sex when using the decision scores of the transgender sex model ($F=6.6$, $p=0.01$), but not sexual orientation ($F=1.6$, $p=0.21$) and no interaction effect ($F=2.0$, $p=0.15$). Alternatively, in order to exclude an effect of sexual orientation on the performed group analyses, a sexual orientation classifier was trained in our sample using a similar approach than already described in the main analysis. Using Neurominer binary classification models were trained to predict sexual orientation (heterosexual vs. bisexual; bisexual vs. homosexual and homosexual vs. heterosexual) on GMV maps. Age did not differ between group ($F_{(2)}=0.04$, $p=0.96$), but sex did ($\chi^2=20.30$, $p<0.001$) with a majority of (biological) men in the heterosexual ($n_{F/M}$ = 12/29) and a majority of (biological) women in the homosexual sample ($n_{F/M}$ = 31/8). The bisexual sample was balanced ($n_{F/M}$ = 21/20). The preprocessing and training process was similar to our main analysis, where neither age nor sex was added as a covariate. As sex was shown to have a major impact on GMV, we were concerned, that a "correction" for sex would mask potential effects of sexual orientation. The results of the three classifier trainings are shown in Supplementary Table 1. None of the neuroanatomical sexual orientation classifier did successfully separate individuals according to their sexual orientation. Hence, we were not able to detect a clear GMV pattern underlying sexual orientation in our sample which justifies that we did not correct for this factor in our main analysis. Compared to the impact of sex on GMV, effects of sexual orientation might therefore be very subtle. Our findings stand in contrast to previous univariate analyses showing structural brain differences in hetero- and homosexual persons (Savic and Lindström 2008). On the other hand, recent findings indicate that this entity might be rather reflected in

structural/functional brain connectivity (Manzouri and Savic 2018b; Safron and others 2018; Savic and Lindström 2008). Despite our efforts to exclude a potential bias of sexual orientation in our analysis, we cannot definitively rule out an effect of sexual orientation on our sex classifier given previous reports of a less pronounced sexual dimorphism in homosexual populations (Burke and others 2017; Manzouri and Savic 2018a; Savic and Lindström 2008) that might overlap with the effects driven by gender incongruence.

SUPPLEMENTARY TABLE 1:

	Group Comparisons	TP	FP	TN	FN	Spec (%)	Sens (%)	FPR	PPV	NPV	AUC	BAC	p-value
Trained models	SexO Classifier Hetero vs. Bi	15	26	15	26	36.59	36.59	63.41	36.59	36.59	0.34	36.59	1.00
	SexO Classifier Bi vs. Homo	25	20	19	16	48.72	60.98	51.28	55.56	54.29	0.51	54.85	0.20
	SexO Classifier Homo vs. Hetero	21	16	25	18	60.98	53.85	39.02	56.76	58.14	0.60	57.41	0.13

Model performances evaluated by means of specificity (Spec), sensitivity (Sens), false positive rate (FPR), positive and negative predicted value (PPV and NPV), area under the receiver operating characteristic (ROC) curve (AUC) and balanced accuracy (BAC). These measures were computed from the confusion matrix containing the number of true positives (TP), false positives (FP), true negatives (TN) and false negatives (FM). SexO, Sexual Orientation; hetero, heterosexual; bi, bisexual; homo. homosexual. P-values are based on a test where the observed prediction performances for the each model was compared to a null distribution of the respective outcome labels by training and cross-validating support vector machine models on n=1000 random label permutations. Model significance was defined at $\alpha=0.05$ as $p=\sum^{n=1000}(BAC_{observed}<BAC_{permuted})/n$.

Hormone levels

Blood sampling was performed before MRI scanning. The analysis of estradiol (E_2) and testosterone (T) was done using quantitative electrochemiluminescence immunoessay method (ECLIA) at the Department of Laboratory Medicine at the Medical University of Vienna (http://www.kimcl.at). E_2 were either not detectable or missing for six subjects, T levels for three individuals. E_2 levels were significantly different across groups (ANOVA, $F=13.42$, $p<0.001$, see Table 1) and post-hoc t-tests corrected for multiple comparison revealed the expected difference in E_2 levels between FC and MC, FC and TW, TM and MC and TM and

TW, respectively (all $p<0.001$). There was no difference in E_2 levels between FC and TM ($p=0.1$), nor MC and TW ($p=0.1$). Similarly, there was a significant difference in T levels across groups (ANOVA, $F=126.67$, $p<0.001$, see Table 1), which was driven by T levels between FC and MC, FC and TW, TM and MC and WM and TW, respectively (all $p<0.001$). There was no difference in T levels between FC and TM ($p=0.1$), nor MC and TW ($p=0.1$).

Multivariate pattern classification analysis (MVPA)

For each binary classification model ((1) sex in cisgender subjects (FC vs. MC); (2) sex in transgender subjects (TM vs. TW); (3) sex-incongruent gender identity in biological females (FC vs. TM) and biological males (MC vs. TW); (4) gender incongruence (FC+MC vs. TM+TW)), the respective GM maps were loaded and adjusted for whole brain TIV outside of the cross-validation structure. No other covariates were added. Then, the following analysis steps were performed within a 10-fold cross validation design both at the outer (CV_2) and the inner (CV_1) levels: Gaussian smoothing was optimized between 0, 4 and 8 mm, then GM maps were scaled voxel-wise to [0, 1] and pruned in order to remove features with zero variance within the training folds of the CV_1. A principal component analysis (PCA) (Hansen and others 1999) was applied to reduce feature dimensionality and discard noisy information. This was performed by retaining those principal components for the subsequent analysis steps, which cumulatively explained 80% of the GM data in each training sample. Then, the single-subject GM volume maps of the CV_1 and CV_2 test samples were projected into the reduced principal components space. Finally, the so obtained PCs scores of our subjects were scaled to [0, 1]. The PC entered a linear C-SVC algorithm (Chang and Lin 2011) that determined the optimal between-group boundary by maximizing the margin between the neuroanatomically most similar subjects of opposite groups (the “support vectors”) (Scholkopf and others 2000). Along with the previously mentioned parameters, the slack (“C-

parameter") was optimized within the CV_1 cycle thus creating an ensemble of 100 optimized C-SVC models to be applied to each CV_2 partition.

Transgender sex classifier

The transgender sex classifier was further investigated as – although significant – the model performed dramatically less well than the cisgender classifier. Using nonparametric Friedman's test to contrast the balanced accuracies of each CV_2 cycle for the cis and transgender sex classifier, a significant difference between both models was determined (χ^2=10, p<0.001). Using a chi^2 test, we compared the distribution of sexual orientation across the correctly and misclassified cases in the transgender sample, but detected no difference (χ^2=2.9, p=0.23). Moreover, levels of T and E_2 were compared by means of a *t* test in the correctly and misclassified cases, similarly yielding no difference (for T: $t_{(50)}$=0.26, p=0.80; for E_2: $t_{(47)}$=-0.10, p=0.92). No available clinical variable demonstrated relationships with decision scores or misclassification rates.

External validation of the sex classifiers

Validation of the cisgender sex classifier in hormone-treated transgender subjects

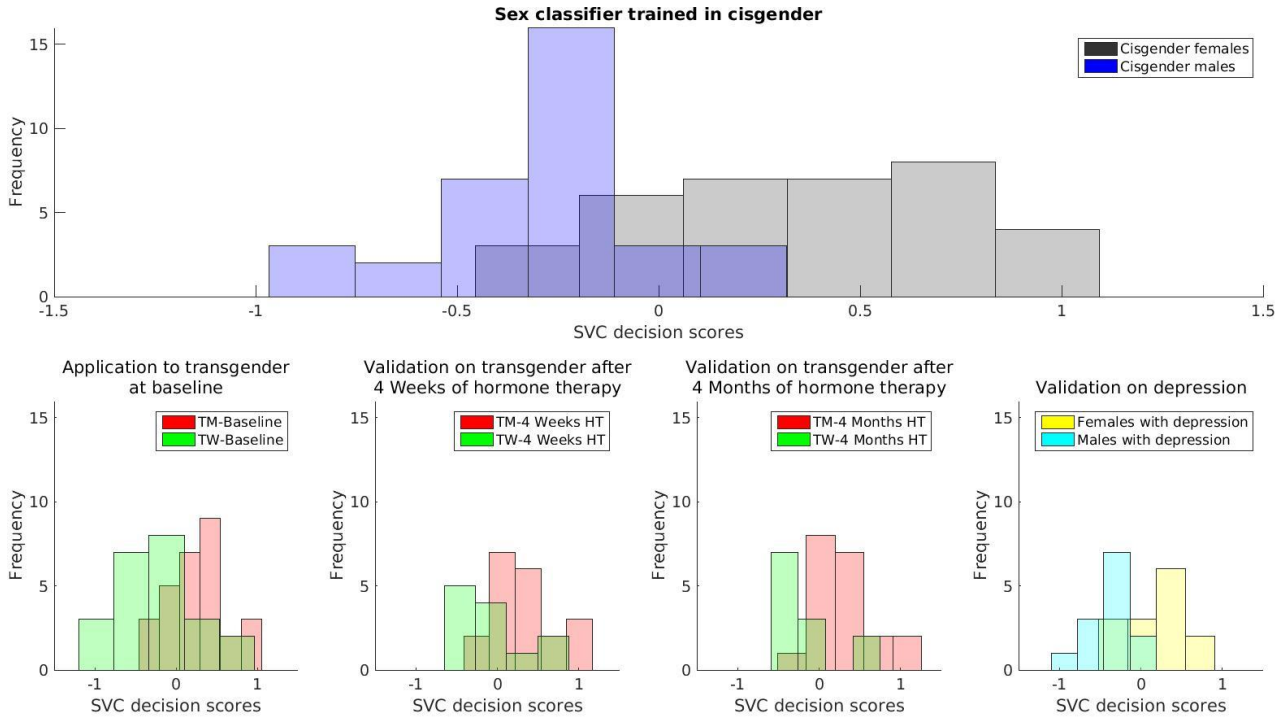
Longitudinal data from 32 transgender subjects were available, namely 20 TM and 12 TW, after starting the respective cross-sex hormonal treatment as 20 subjects discontinued their study participation before starting hormonal treatment. TM subjects received either 1000 µg testosterone undecanoate every 12 weeks (Nebido 250 mg/ml, intramuscular) or 50 mg testosterone daily (Testogel 50mg/5ml, transdermal). Additionally, either 10-15 mg lynestrenol (Orgametril 5 mg, oral) or 75 µg desogestrel (Cerazette 75 µg, oral) were administered daily if menstruation still persisted. TW participants received 50 mg cyproteroneacetate (Androcur 50 mg, oral) and estradiol hemihydrate 4 mg (Estrofem 2 mg,

oral) daily. Alternatively, some subjects were treated with a transdermal application of estradiol hemihydrate (Estrogel 75 mg/1.25 mg, transdermal). Measurements were performed on the same MRI scanner at each time point.

The cisgender sex classifier model was tested on this subset of transgender individuals as described in the main text, showing a significantly worse performance at both time-points with a BAC of 75.0% (Spec=75.0%, Sens=75.0%, McNemar's $\chi 2$=12.64, p<0.001) in the transgender sample after four weeks and a BAC of 75.0% (Spec=75.0%, Sens=75.0%, McNemar's $\chi 2$=12.64 p<0.001) after four months of treatment compared to baseline before treatment (BAC=76.2%). Using a classical mixed modelling approach with Satterthwaite's method, treatment effects were tested as function of time. This was performed by evaluating changes in the decision scores separately for women and male participants, given that the obtained hormonal treatment was different for each sex. Results indicate that there was a significant effect of therapy across time in TW ($F_{(1,23)}$=12.29, p<0.001) whereas no effect for TM participants ($F_{(1,39)}$=1.597, p=0.21) was observed.

The distribution of the decision scores generated by the cisgender model and by its application to the transgender (baseline, after four weeks and four months of hormonal treatment) and depressed subjects is displayed in Supplementary Figure 1. While the distribution patterns in females and males are very distinct in cisgender subjects (Supplementary Figure 1A) and when applied in the depressed sample (Supplementary Figure 1E) with only low overlap, separability of untreated transgender females and males based on the cisgender sex classifier is significantly impaired with a more pronounced overlap and a shift of TW decision scores in the positive range (Supplementary Figure 1B). This effect is even stronger after four weeks and four months of cross-sex hormonal treatment with total a dilution of a distinct male pattern based on decision scores and less values in the negative range (Supplementary Figure 1C and 1D).

SUPPLEMENTARY FIGURE 1: Distribution of the decision scores of the cisgender sex classifier



Upper histogram (A) displays the distribution of the decision scores generated by the cisgender sex classifier model. Cisgender males are shown in blue, cisgender females in black. In the lower row, the distribution of the decision scores is shown when applying the cisgender sex classifier on hormone-naïve transgender subjects at baseline (B), after four weeks (C) and four months of cross-sex hormone treatment (D), as well as in an external sample of depressed patients (E). In (B,C,D) TM subjects are shown in red and TW subjects in green, in (E) depressed females are displayed in yellow and depressed male in light blue. TM transgender man, (syn. female-to-male transgender); TW transgender woman (syn. male-to-female transgender); HT hormone therapy; SVC support vector classification.

Validation of the sex classifiers in a sample with major depression

Furthermore, the cis- and transgender sex classifier models were adopted on an independent sample of medication-naïve patients (N=27, F/M=14/13) suffering from major depression (17-item Hamilton Depression Rating Scale Score=22.78±4.97) with a comparable age (28.30±9.82). Depressed subjects were measured on a Siemens Biograph mMR PET/MR scanner (MPRAGE sequence, TR = 3000ms, TE = 4.2 ms, 160 x 240 x 256 matrix, 1.1 x 1.0 x 1.0 voxel size).

Univariate analysis

Image preprocessing

As described in the main manuscript, image preprocessing was done using the CAT12 toolbox (Gaser and Dahnke 2009) for SPM12 (Wellcome Trust Center for Neuroimaging, Statistical Parametric Mapping, version 12, http://www.fil.ion.ucl.ac.uk/spm/software/spm12/) in MATLAB R2015a (The MathWorks, Inc, 2015, http://www.mathworks.com/). All steps were performed identically to the preprocessing performed for the MVPA. Also, no an additional smoothing was chosen based on parameter optimization performed in NeuroMiner, where a smoothing of 0 mm was chosen in the vast majority of the generated models.

Statistical analysis

Conforming to the MVPA analysis, the warped and modulated GM volume maps entered a full factorial model as specified in SPM12 including sex and cis/transgender identity as factors. The check for design orthogonality with TIV as covariate showed a high correlation of TIV with sex, wherefore we chose the alternative approach using global scaling with TIV in the full factorial model. Upon whole brain model estimation, F contrasts were computed to assess main effects and interactions of sex and cis/transgender identity. T contrasts were calculated based on the group comparisons performed in NeuroMiner (FC vs. MC, TM vs. TW, FC+TW vs. TM+MC, FC vs. TM, MC vs. TW, FC+MC vs. TM+TW). Statistical maps were considered significant if they survived FDR (False Discovery Rate) correction for multiple comparisons ($p_{FDR} \leq 0.05$). Anatomical labeling of significant clusters was performed using the xjView toolbox (http://www.alivelearn.net/xjview/).

Results

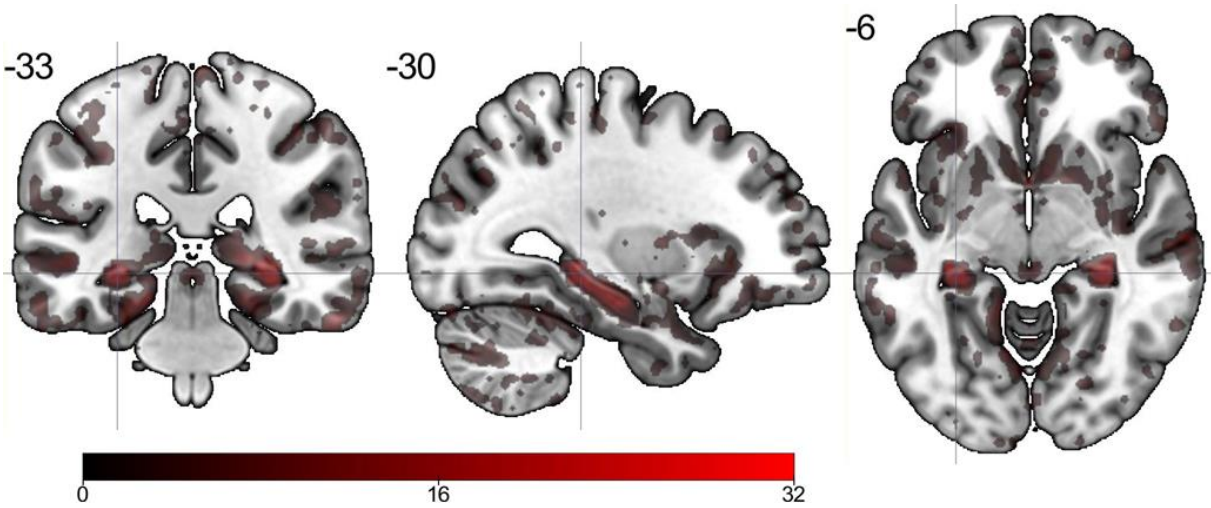
We found a significant main effect of sex in our sample in the left (F=31.84, $p_{FDR\text{-}corr}$=0.043, see Supplementary Table 2 and Supplementary Figure 2), the right hippocampus (F=31.13, $p_{FDR\text{-}corr}$=0.043), the left fusiform gyrus (F=29.50, $p_{FDR\text{-}corr}$=0.043) and the left caudate (F=29.48, $p_{FDR\text{-}corr}$=0.043). T-contrasts showed an increased GMV in the female bilateral hippocampus when comparing cisgender females and males (left: *t*=5.45, $p_{FDR\text{-}corr}$=0.049; right: *t* =5.36, $p_{FDR\text{-}corr}$=0.049, see also Supplementary Table 2). There was no significant difference when contrasting groups of divergent gender identity or when comparing cis- and transgender individuals.

SUPPLEMENTARY TABLE 2: Univariate analysis

Contrast	Anatomical Region (AAL)	Cluster size	peak				MNI coordinates (mm)		
			p FDR-corr	F/T	Z	p uncorr	x	y	z
Main Effect Sex									
	Hippocampus_L	61	0.043	31.84	5.17	<0.001	-33	-30	-6
	Hippocampus_R	68	0.043	31.13	5.11	<0.001	33	-24	-12
	Fusiform_L	14	0.043	29.50	4.99	<0.001	-21	-36	-18
	Caudate_L	16	0.043	29.48	4.98	<0.001	-6	6	6
	Caudate_L	26	0.052	27.92	4.86	<0.001	0	-15	3
FC > MC									
	Hippocampus_L	73	0.049	5.45	5.14	<0.001	-30	-21	-15
	Hippocampus_L	22	0.049	5.36	5.06	<0.001	-21	-39	-15
	Hippocampus_R	85	0.049	5.30	5.01	<0.001	27	-33	-3

Table summarizing the results of the full factorial model performed in SPM, corrected for multiple comparisons (FDR, false discovery rate). A main effect of sex could be detected in the bilateral hippocampus, the left fusiform gyrus and the left caudate. Regional grey matter volume (GMV) differences were driven by higher GMV in FC vs. MC in this brain area. There was no difference in GMV when comparing groups of divergent gender identity nor when comparing cis- and transgender individuals. FC, female cisgender; MC, male cisgender.

SUPPLEMENTARY FIGURE 2: Main effect of sex on GMV



Main effect of sex (F=31.84, $p_{FDR\text{-}corr}$=0.043) on grey matter volume (full factorial model in SPM corrected for TIV) overlaid on the single subject MNI template using the software MRIcroGL (http://www.cabiatl.com/mricrogl/). The cross-hair is situated on the left hippocampus.

Discussion

Compared to our MVPA where we detected a widespread neuroanatomical pattern underlying sex, the only sexually dimorphic brain regions detected in our sample using univariate statistics were the hippocampus, bilaterally, the left caudate and fusiform (see Supplementary Table 2), which are also encompassed in the pattern generated in the MVPA and correspond to the regions chosen in both (cis- and transgender) sex classifier models (see Figure 2). Interestingly, the hippocampus has been intensively investigated in regard to sex-specific size differences as this brain region was shown to be relevant in the pathophysiology of various psychiatric disorders that are more prevalent in women and commonly related to stress and hippocampal atrophy (Cahill 2006; Filipek and others 1994; Giedd and others 1996; Goldstein and others 2001; Sapolsky 2002). Most studies claim that the female hippocampus is disproportionally large, which is in accordance with our results (Persson and others 2014). However, a meta-analysis reported no sex-related difference in this region (Tan and others 2016) and claimed that differences might rely on methodological issues and head size

correction (Perlaki and others 2014). Also, the hippocampus was not part of the region-of-interest approach chosen by Hoekzema et al. in their MVPA regarding sex effects (Hoekzema and others 2015). Still, the significant contrast detected in our univariate analysis, namely the one comparing cisgender women and men (FC vs. MC), which is supported by our most discriminative MVPA model (see Table 2) that considered correction for TIV is highly indicative of the fact that the hippocampus exhibits strong sexual dimorphic characteristics.

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