REFINING PREVENTION: GENETIC AND EPIGENETIC CONTRIBUTIONS

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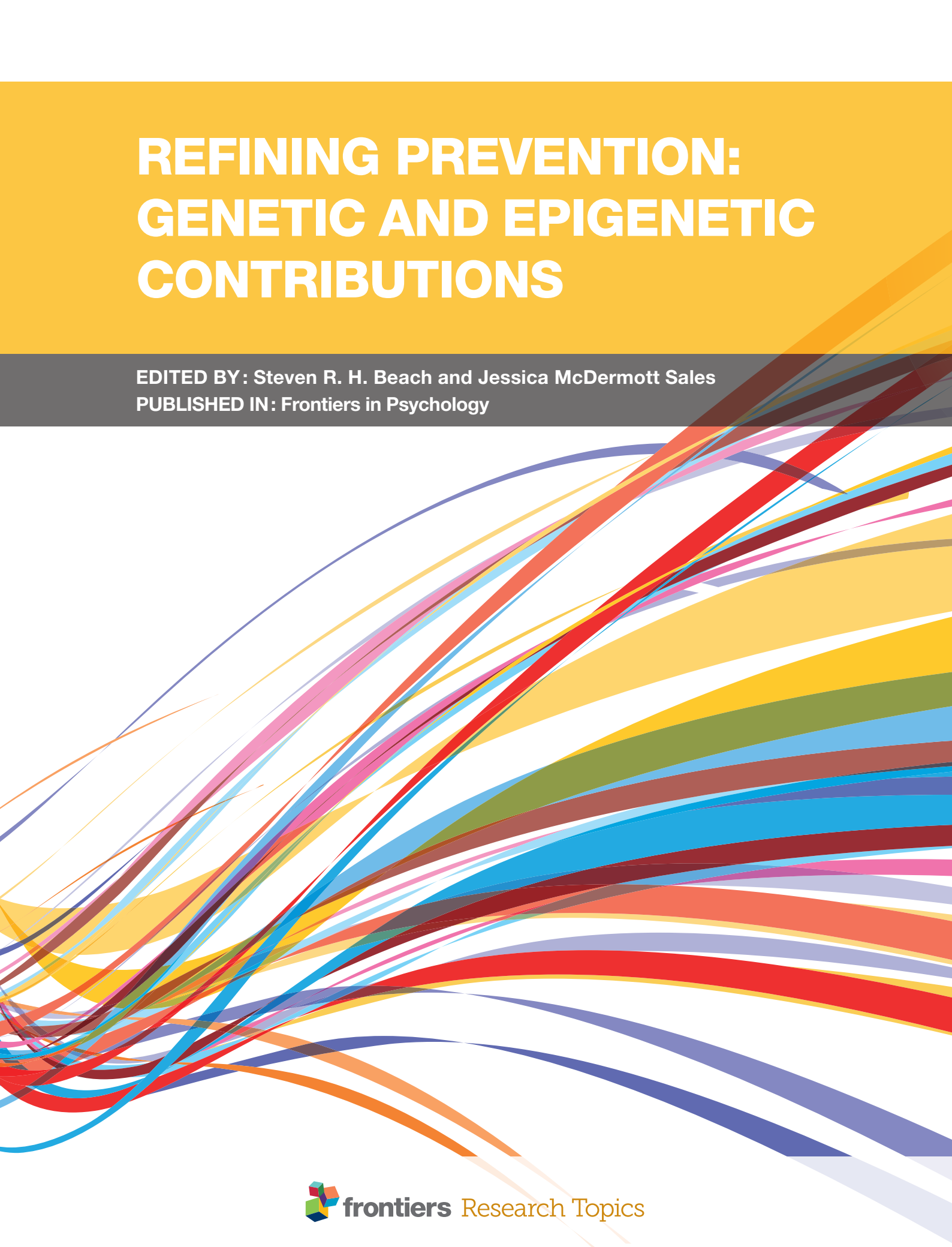

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REFINING PREVENTION: GENETIC AND EPIGENETIC CONTRIBUTIONS

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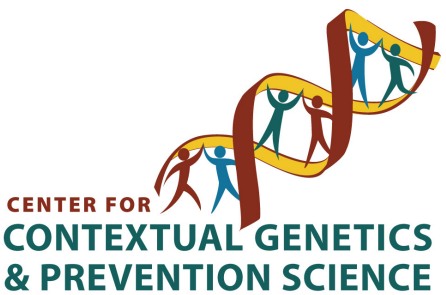

Center of Family Research.
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Currently, most prevention efforts are framed as universal interventions. However, despite the demonstrated efficacy of many prevention programs, variability in response is the rule with some participants responding very little and others accounting for the bulk of the positive impact of the program. Better understanding the processes associated with better and worse response to prevention is a critical first step in refining and adapting existing programs, or alternatively designing new prevention programs with enhanced outcomes. Because vulnerabilities to substance use, emotional problems, risky sexual behavior and other behavioral problems are influenced by a combination of environmental, genetic, and epigenetic factors, mediated in part through psychological processes (Kreek et al., 2005; Rutter et al., 2006), the study of genetic and epigenetic vulnerability and susceptibility factors provides an important starting point for efforts to address this critical need.

A growing body of research on differential genetic susceptibility indicates that efforts to enhance prevention impact may benefit from consideration of the contribution of individual genetic differences to treatment response (Brody et al., 2013). In addition, the recent expansion of genetic research to include a focus on epigenetic change provides considerable promise for the development of indicated prevention and individually tailored prevention efforts. However, before this promise can be realized, a number of theoretical and practical challenges remain. Thus, through this special section, we provide a foundation for a new era of prevention research in which the principles of prevention science are combined with genomic science.

In the current special section we bring together authors to deal with genetic and epigenetically driven processes relevant to depression, substance abuse, and sexual risk taking. Together they comment on, and provide data relevant to, assessment, research and statistical methods, The

papers help to inform the development of a new generation of prevention programs that go beyond universal programs and sensitively target key processes while providing greater precision regarding prediction of population-level impact.

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EDITORIAL
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Editorial: Refining Prevention: Genetic and Epigenetic Contributions

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Keywords: prevention, translation, genetics, epigenetics, substance use, mental health

The Editorial on the Research Topic

Refining Prevention: Genetic and Epigenetic Contributions

The current series of articles was designed to capture ongoing translational activities linking genetic and epigenetic research to enhancement of prevention and treatment efforts. Better understanding the processes associated with better and worse response to a range of environmental causes and to preventive interventions is a critical first step in refining and adapting existing prevention programs, or alternatively designing new prevention programs with enhanced outcomes. In the current series of papers we address translational issues from several directions. Our first three papers highlight methodological and conceptual innovations with the potential to guide researchers in new directions and maximize the impact of ongoing G and GxE research. The next set of four manuscripts provide an excellent example of informative empirical tests of G and GxE effects on key symptoms, mediators, and outcomes. This type of work highlights the potential for genetically informed research to illuminate variable base rates of target behavior, build upon GWAS results to test specific, theory driven hypotheses, and highlight populations with greater sensitivity to common environmental stressors. Our last two contributions point to future directions, the first examining innovative strategies that may be transportable to many contexts, allowing researchers to develop broad coalitions to examine genetic effects on behavioral health, and the second introducing epigenetic variables as a way to further inform preventive research and illustrate the key role of family environments in laying the groundwork for young adult health. Together the series provides a useful overview of strategies and approaches as well as important insights for future translational efforts. To guide examination of specific manuscripts, we briefly characterize each below.

The first paper by Howe and colleagues describes a new research design and analytic approach, the baseline target moderated mediation (BTMM) design, and characterizes its utility for enhancing the investigation of genotypic moderators of prevention trial results, and strengthen the potential for such investigations to lead directly to treatment innovation. Ultimately, this approach will help researchers more readily identify subgroups most likely to respond, help explicate the mechanisms involved, and sidestep many of the potential difficulties associated with genetic selection in primary prevention.

Our second paper by Philibert and colleagues introduces a new epigenetic measurement tool to advance the examination of preventive intervention efficacy as well as treatment efficacy in the context of smoking reduction and prevention. They identify methylation status at a CpG locus in the aryl hydrocarbon receptor repressor, cg05575921, as a sensitive and specific indicator of smoking status and history in young adults, suggesting that utilization of this locus in a methylation-based diagnostic scheme could be a great help in documenting prevention effects and improving the characterization of response to treatment of smoking, and smoking related disorders.

Our third paper by Gray and MacKillop provides an overview of the literature on delayed reward discounting (DRD), an index of how much an individual devalues a future reward based

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on its delay in time. This novel construct is explicated in part by examination of genetic and environmental correlates. Given its robust association with various drugs of abuse, DRD appears to be worthy of investigation at a more general level as a novel and promising drug abuse prevention target.

The second section of our series begins with a paper by Obasi and colleagues, who find that perceived stress and alcohol consumption have a deleterious effect on HPA-axis functioning. In particular, perceived stress and alcohol consumption disrupt the cortisol awakening response, an effect that is superimposed on genetic influences. Likewise, the next paper in this section by Nikolova and colleagues uses a sophisticated mix of methods to examine the effect of a SNP previously linked to depression by GWAS investigation. They show that that carrying the A allele of *FREM3* may be implicated in blunted amygdala reactivity, slower reaction times in face-matching, and marginally slower performance on the Tail Making Test, suggesting possible brain mechanisms linking genotype to outcomes. Our sixth paper by Windle and Mrug uses a GxE approach to explicate the role of disrupted attachment on depression. In particular, they show that young adult females who experienced parental divorce during adolescence and have the "GG" oxytocin genotype had substantially greater risk of depressive symptoms compared to those experiencing parental divorce but carrying an "AA" or "AG" genotype. Our seventh paper by Sales and colleagues explores the effect of carrying the "*s*" allele at the 5HTTLPR, and its effect on increased reactivity to experiences of discrimination, experiences that are all too common among young adult African-Americans. They find that high levels of racial discrimination are significantly associated with greater odds of high depressive symptoms only for participants with the "*s*" allele, with an interaction predicting depressive symptoms among African–American adolescent females.

Our eighth paper in the series by Dick and Hancock describes the "Spit for Science" project, a remarkably successful project conducted at a large, public, urban university in the United States that was able to unify efforts across campus to address problematic college student substance use and mental health issues, creating large new data sources for examining genetic contributions to behavioral health outcomes in the process. This program provides a template for the type of creative scientific paradigms that may be useful, and indeed essential, if efforts to personalize behavioral health interventions are to move forward.

The ninth and final paper by Beach and colleagues illustrates the potential power of the "epigenetic" measurement tools that have recently become available to family and prevention science researchers. These tools provide an opportunity to identify potential biological mechanisms linking family contexts and health or health behavior outcomes. This paper suggests that examination of epigenetic mechanisms offer considerable promise for family researchers to further expand their etiological models. In particular, the authors find that epigenetic regulation of a key inflammatory factor, Tumor necrosis factor (TNF) is associated with early family environment, and mediates the association of earlier parenting with young adult health outcomes years later.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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METHODS
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Translating Genetic Research into Preventive Intervention: The Baseline Target Moderated Mediator Design

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In this paper we present and discuss a novel research approach, the baseline target moderated mediation (BTMM) design, that holds substantial promise for advancing our understanding of how genetic research can inform prevention research. We first discuss how genetically informed research on developmental psychopathology can be used to identify potential intervention targets. We then describe the BTMM design, which employs moderated mediation within a longitudinal study to test whether baseline levels of intervention targets moderate the impact of the intervention on change in that target, and whether change in those targets mediates causal impact of preventive or treatment interventions on distal health outcomes. We next discuss how genetically informed BTMM designs can be applied to both microtrials and full-scale prevention trials. We use simulated data to illustrate a BTMM, and end with a discussion of some of the advantages and limitations of this approach.

Keywords: prevention research, genes, gene-environment interaction, research design, moderation, mediation

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INTRODUCTION

As advances accumulate in molecular and behavioral genetics, there is growing interest in translating those findings into practical application. Recent findings from prevention studies bolster this idea. For example, candidate gene variability moderates the impact of family-based interventions designed to reduce risk for adolescent substance use (Brody et al., 2013b) or for early childhood aggression (Bakermans-Kranenburg et al., 2008). This has led to suggestions that genetic moderation may help identify subgroups more likely to benefit from preventive intervention, or alternatively identifying those who will not benefit from preventive intervention. However, we view this outcome as highly unlikely, due to the complex picture of gene-environment dynamics that has emerged over the past decade (Brody et al., 2013a). As **Figure 1** illustrates, genetic variation interacts with environmental conditions at several points in the pathway from polymorphic variation to phenotypic behavior, and these pathways also evolve over the course of development. Because preventive interventions target only a subset of these pathways and genetic variation typically moderates the effects of only some specific aspects of preventive intervention, there are always likely to be untargeted pathways and/or pathways not genetically moderated that also contribute to future risk. From this perspective genetically informed research is best viewed as a way to elaborate pathways of risk, provide a window on risk mechanisms, and identify pathways not currently targeted by preventive interventions that may be promising targets for the next generation of prevention trials. Against this backdrop, we suggest that there is a need for a flexible framework to guide translation, taking us from genetic moderation of preventive intervention effects to prescriptive implications for prevention.

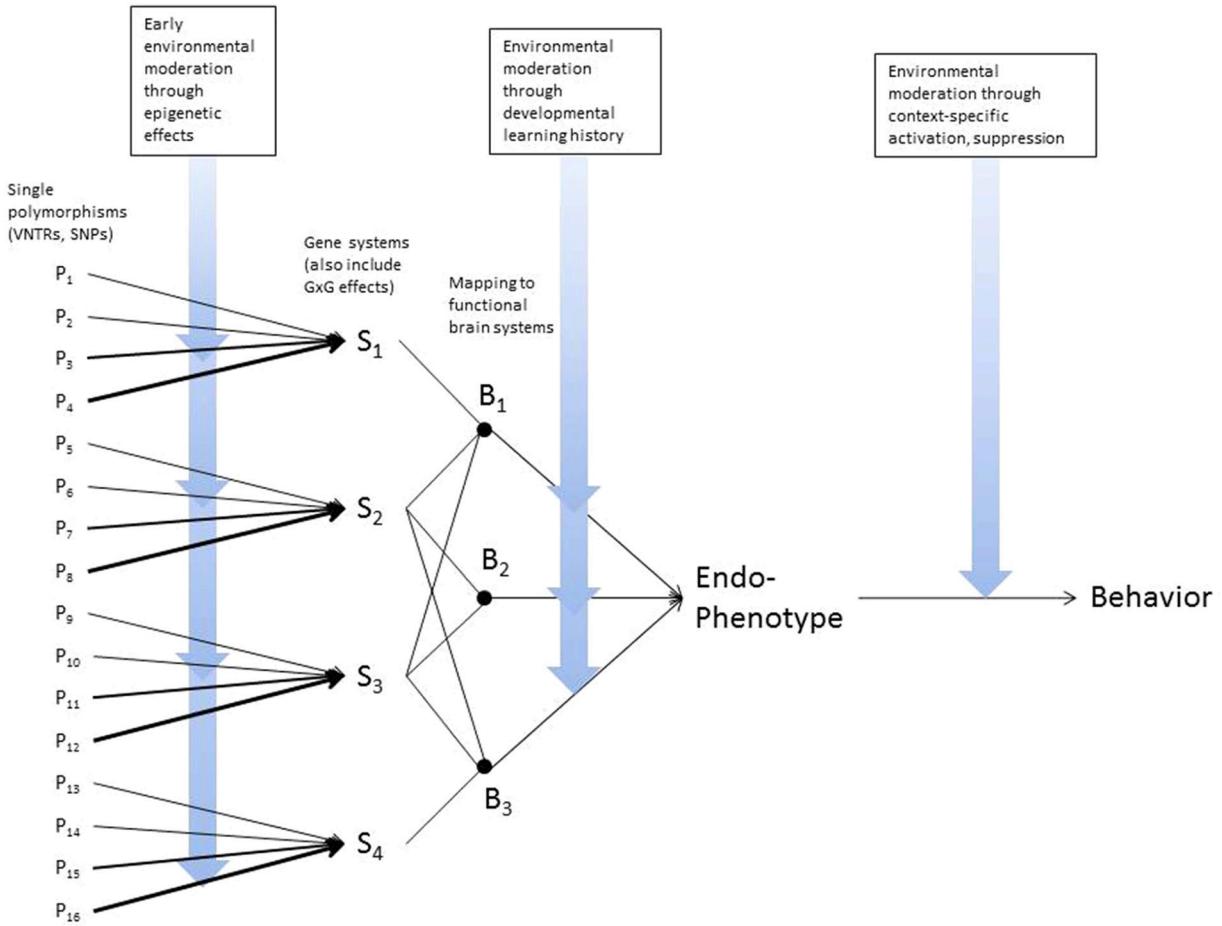

FIGURE 1 | Dynamic interplay of genes and environments across various levels of analysis.

In this paper we present and discuss a novel research approach, the baseline target moderated mediation (BTMM) design, that holds substantial promise for advancing this agenda. In particular, we suggest that this design will be more likely to help us answer the general question of which preventive interventions work best for what groups, and why that might be so. We begin by discussing how genetically informed research on developmental psychopathology can be used to identify potential intervention targets. We then describe the BTMM design and its promise in determining whether those proximal targets mediate causal impact of preventive interventions on distal health outcomes, through combining tests of both moderation and mediation in longitudinal designs. We next discuss how genetically informed BTMM designs can be applied to both microtrials and full-scale prevention trials. We end with a discussion of some of the advantages and limitations of this approach.

MALLEABLE RISK AND PROTECTIVE MECHANISMS AS PROXIMAL TARGETS

Prevention science has a long history of using findings from developmental psychopathology to identify proximal intervention targets. Prevention scientists focus on evidence concerning mechanisms that increase risk for future emotional or behavioral disorders, as well as mechanisms that protect people in the face of such risk. Risk and protective mechanisms must also be placed in developmental context, given evidence that risk trajectories can start early and be modulated by events occurring through childhood and adolescence. And for such mechanisms to be of use for prevention scientists, they must be malleable enough to be altered by intervention technologies (Coie et al., 1993). For example, the Strong African American Families program identified a set of protective mechanisms that could be influenced by working with parents on active parenting of adolescents, and working with youth to help them develop a future orientation and to formulate self-care strategies (Brody et al., 2015). These activities were designed to change cognitions involving intentions to use drugs and positive images of drug-using peers, as well as enhancing self-regulation skills, based on developmental research linking these mechanisms to escalation of drug use.

A second example, the Sources of Strength program, identified peer leader modeling of positive coping behavior as a protective mechanism that can spread through adolescent social networks (Wyman et al., 2010). This program trained peer leaders

to provide positive-oriented communications to high school classrooms, sharing narratives about their own use of healthy coping resources as a proximal target for reducing future risk of suicidal behavior in those peer communities (Petrova et al., 2015).

GENETICALLY INFORMED PROXIMAL TARGETS

How can genetic research inform our understanding of malleable risk or protective mechanisms? Within a counterfactual framework (Rubin, 1974), genes are not causes. Or to be more precise, polymorphic variation in candidate genes across a population involves a stable, unchanging background factor (at least until genetic manipulation through gene therapy is possible). Although such factors cannot be considered causes, they can act as causal modifiers. Variation in candidate genes can therefore index variation in the causal structure underlying risk and protection (also referred to as causal effect heterogeneity: Brand and Thomas, 2013), where that structure involves one or more factors that can be manipulated. These malleable factors are of paramount importance for prevention science, as they reflect key targets for interventions designed to prevent future distress or disorder.

We suggest that genetically informed research can be useful for prevention science, to the degree that it tells us something about variation in the impact of risk or protective mechanisms. This can happen in several ways. Experimental studies can test not only whether a specific manipulation can alter a mechanism, but also whether those effects differ depending on genotype. Fox et al. (2011) used a gene-manipulation interaction design to study negative attention bias, a risk factor for anxiety disorder. They were able to modify attention bias in participants having less efficient alleles of the serotonin transporter gene, but not in those with the more efficient allele. Given that participants with both types of alleles are equally at risk for anxiety disorders (Blaya et al., 2007; Gressier et al., 2013; Mak et al., 2015), this suggests that negative attention bias may not be a relevant risk factor for the group carrying the more highly efficient allele. Fox et al. (2011) suggest that the latter group is less prone to negative attention bias in general because they are less influenced by negative experiences over the course of development. This implies that genetic moderation of change in attention bias was mediated by baseline levels of attention bias (emerging through earlier development), with those low in initial attention bias demonstrating less change in subsequent bias than those with initially higher bias [unfortunately Fox et al. (2011) did not test for this effect]. As we discuss later, moderation of effects by baseline target levels may be common in prevention trials, given that change in a target is less likely when an individual or family already has high levels of a protective target or low levels of a risk target.

Fox and other investigators (e.g., Lonsdorf et al., 2009) have used this design to target behavioral or cognitive risk mechanisms, but other genetically informed studies have focused on environmental risk mechanisms. These include observational studies of gene-environment correlation and gene-environment interaction. Several studies have found that specific parent (Klahr et al., 2015) and child (Kryski et al., 2014) genes are associated with parental warmth and hostility, two factors often targeted in interventions to prevent future substance use or other problem behaviors. A rapidly expanding set of studies also document that the association between parenting behavior and child outcomes is moderated by specific child genes including polymorphisms in BDNF (Chen et al., 2015), DRD4 (Cho and Kogan, 2015), and COMT (Sulik et al., 2015). Both sets of findings suggest that preventive interventions that change parenting behavior may have differential effects on this proximal target depending on baseline levels of parenting, which can mediate earlier gene-environment interplay for both parent and child.

Finally, a growing number of studies have included genetic assessments in randomized prevention trials, allowing for tests of whether specific genes moderate the impact of interventions on both proximal targets (Brody et al., 2015) and distal outcomes (Brody et al., 2013b). In most cases, the intervention is found to have less or even no impact on genetically defined subgroups. We suggest that these will often reflect situations where baseline levels of targets involve those with lower risk (or higher protective potential).

In summary, the accelerating research effort incorporating several types of genetically informed designs can point to important potential moderators of intervention impact, guiding us in identifying what works for which people. However, we suggest that this will often be most useful when it identifies malleable proximal targets and tests whether baseline levels of those proximal targets act to moderate intervention effects on both change in proximal targets and change in distal outcomes.

BASELINE PROXIMAL TARGETS AS MODERATORS

There is growing interest in studying potential moderators of preventive intervention, as a means of learning which interventions work with which people, and under what conditions. Investigators often start with the "usual suspects," focusing on broad demographic characteristics such as gender, economic condition, or ethnicity. Rather than focusing on such broad factors, we suggest that theoretically "active" moderators, i.e., baseline levels of targeted mediators, will often be more promising to pursue. Consideration of malleable targets that mediate impact on outcomes suggests the strong possibility that baseline levels of the targeted mediator will moderate the impact of the intervention. More broadly, because preventive intervention research is guided by etiologic theories that identify which developmental mechanisms to target and by action theories that identify mechanisms that change those specific targets (MacKinnon et al., 2002), assuming equivalent responsiveness across participants, the impact of the intervention should vary across participants depending on how much room there is for change from the baseline level of the targeted mechanism. Those individuals or families who have higher levels of some targeted risk factor or lower levels of some protective factor have more room to improve, and so, all other things being

equal, have greater room for potential improvement in targeted mediators and so greater potential for impact of the intervention on distal health outcomes. Those who begin at lower risk or have more of the protective resource have less room to improve. In more formal terms, baseline levels of an active target should moderate the impact of the intervention on subsequent change in that target.

There are already examples illustrating the basic effect predicted by the BTMM. In the Familias Unidas program family-based interventions were specifically designed to increase positive communication between parents and adolescents in Hispanic families. Accordingly, positive communication was the targeted mediator. In a study that combined data from three randomized trials of the Familias Unidas program, Perrino et al. (2014) found that baseline communication moderated the impact of the intervention, such that those families in the intervention group with poorer communication showed greater increases in positive communication compared to those who began with better communication, while the control group showed no changes in communication regardless of baseline level. Another prevention program working with Mexican American families, the Bridges/Puentes school-based program demonstrated similar effects for several targeted parent behaviors including harshness, positive reinforcement, and monitoring, as well as adolescent active coping and school involvement (Gonzales et al., 2012). These findings were also strengthened through indexing change in the target from baseline to post-test, in these cases using autoregressive modeling.

EXPANDING TESTS OF PROXIMAL TARGETS AS MEDIATORS

If the etiologic theory guiding selection of proximal targets is correct, than change in targets should in turn lead to change in more distal outcomes involving behavioral or emotional health. Testing mediational pathways will therefore provide further evidence concerning the validity of both the action and the etiologic theories. This requires that we develop intervention trial designs that allow us to detect changes in both proximal targets and distal outcomes, and that we use statistical techniques that allow for rigorous tests of these theories. Such tests require longitudinal designs that track variation in both target and outcome over developmentally appropriate time periods. Designs employing at least three measurement occasions (baseline, post-test, and follow-up) allow for direct modeling of change in both targets and health outcomes, where change in the former (between baseline and post-test) precedes change in the latter (between post-test and follow-up). Perrino et al. (2014) found that changes in family communication were influenced by the intervention, and in turn post-test communication mediated the impact of the intervention on changes in adolescent internalizing, with better communication associated with decreasing slopes in internalizing as indexed by growth curve models. Gonzales et al. (2012) found evidence of target mediation for several outcomes including adolescent substance use, externalizing, internalizing, and school performance, although not all targets acted as mediators.

An expanded mediational design allows for statistical tests of lagged change-to-change mediation (where change in the proximal target leads to subsequent change in the distal outcome). This model increases plausibility that targeted mechanisms are having a causal impact on outcomes. Modeling the association between change in target and change in outcome is more consistent with a counterfactual account of cause (Morgan and Winship, 2007), particularly when the change in target precedes the change in outcome. Such models allow for testing causal precedence through cross-lagged regression. This method also tests whether earlier changes in the distal outcome may precede changes in the target. If this is not found we can rule out alternate hypotheses that change in outcomes precedes change in targets.

Such models may be of particular use when risk for behavioral or emotional disorders are low at the time of intervention but increase over later stages of development. Most studies we have encountered do not employ true lagged change-to-change models, but rather test whether the target measured at post-test is associated with autoregressive change in the outcome. True lagged change-to-change mediation requires some way of indexing change in target as well as outcome, and can be achieved through the use of latent change models (McArdle, 2009). These models are able to estimate change across two measurement occasions, and therefore require as few as three waves of data to test a change-to-change hypothesis.

COMBINING MODERATION AND MEDIATION IN THE BTMM

If the mechanism we target in a preventive intervention is a true risk or protective mechanism for the distal health outcome, then not only will it mediate the impact of the intervention on that outcome, but that mediation effect itself will vary by target baseline level. This pattern is defined as moderated mediation (James and Brett, 1984). Judd and Kenny (1981) were among the first to apply this concept to the evaluation of intervention trials. As an example, Perrino et al. (2014) demonstrated that the strength of the mediation effect for family communication on adolescent internalizing was highest for those families with the poorest communication at baseline, and decreased as baseline communication quality increased. When combined with change-to-change mediation, moderated mediation provides the basis for the full BTMM design. **Figure 2** provides a directed graph for a BTMM model that employs lagged latent change. As we noted earlier, when studying whether a preventive intervention works differently for different subpopulations, most investigators have focused only on general population characteristics such as gender or ethnicity. We suggest that focusing on baseline levels of intervention targets will be more productive in identifying key moderators of intervention effect, and will provide more relevant information for tailoring next generation prevention trials.

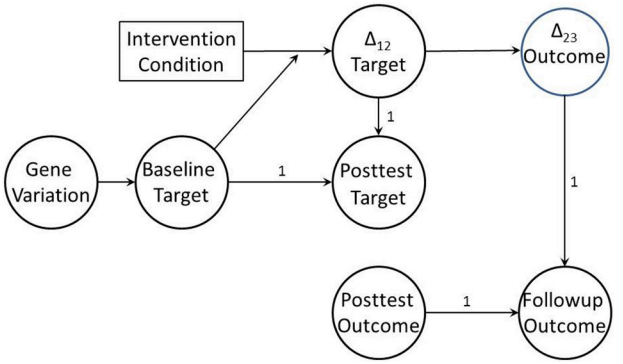

FIGURE 2 | Baseline target moderated mediation model with lagged change-to-change mediation. Baseline target, posttest target, posttest outcome, and follow-up outcome are all measured as latent variables. Δ_{12} is a latent variable referencing change in target variable from pretest to posttest; Δ_{23} is a latent variable referencing change in outcome from posttest to follow-up.

EXPANDING THEORETICAL PRECISION BY IDENTIFYING MULTIPLE PROXIMAL TARGETS

Most preventive interventions target more than one risk or protective mechanism in order to maximize impact. When multiple proximal targets are measured, different BTMM models may be necessary depending on whether targets are independent, clustered, or involve chained mediation. Independent targets would have unique and separable impact on health outcomes. In this case etiologic theory would predict that each baseline target would moderate effects only for change in that specific target. The advantage of such a model would be the opportunity to demonstrate specificity by showing both the moderating effects of baseline target levels on change in that target, as well as the lack of moderating effect attributable to baseline levels of other targets. This model has the potential to be powerful and persuasive, but we have been unable to locate any prevention studies employing this approach, probably because etiologic theories do not often identify such independent mechanisms.

Clustered targets involve variables that covary, with proximal targets often influencing each other or jointly indexing the operation of a dynamic system that an intervention is attempting to influence. For example, the Bridges program targeted parent behavior, but also focused on the quality of interactions between parents and adolescents, given that parent harsh behavior covaried with adolescent externalizing (Wong et al., 2014). Mother-adolescent conflict was found to mediate the effect of the intervention on reductions in later adolescent internalizing and externalizing problems (Jensen et al., 2014). This study used a single measure to index the combined effects of parent and adolescent behavior. An alternative approach would be to model the variance shared by a set of proximal targets as a latent variable, and employ the latent variable to estimate both baseline target levels and subsequent change in the targeted mechanism within a BTMM design. For example, Howe et al. (2004), in a study of couples facing unemployment, found that partner reports of support and undermining were strongly correlated. They hypothesized that these variables indexed the quality of dyadic interaction, and used a latent variable model to study the association of the couple-level variable with economic and employment stressors.

Sets of clustered targets may also reflect underlying risk classes that benefit from different components of the preventive intervention. Methods such as latent class analysis can uncover such classes when applied to a set of baseline targets. BTMM analyses using risk classes as the baseline moderator can then be applied to proximal target variables in order to test whether different classes show differential impact due to changes in different sets of proximal targets. Findings of differential effect can then guide development of adaptive interventions that emphasis different proximal targets for different participants.

Chaining (or mediational cascade) involves a series of sequential mediators that transmit the effects of preventive intervention to distal health outcomes. Chaining is common in developmental theories, and is often essential to understanding the effects of early risk or protective mechanisms on later outcomes. This can be of particular importance for health conditions that emerge only at later points in development. For example, McClain et al. (2010) used longitudinal follow-up of a preventive intervention for children of divorced parents to demonstrate that early changes in parent–child relationships were associated with reductions in internalizing, which in turn were associated with reduced risk for externalizing in later adolescence.

GENETICALLY INFORMED BTMM DESIGNS

Baseline target moderated mediation designs can be informed by genetic data in several ways. There has been a recent spate of laboratory experiments demonstrating genetic moderation of highly proximal target response to environmental manipulation. For example Lonsdorf et al. (2009) found that 5-HTTLPR polymorphisms moderate the impact of fear stimuli on fear conditioning but not extinction, while COMT val15met polymorphisms moderated extinction but not conditioning. Growing knowledge of how genes or gene systems are involved in developmental pathways can provide important leads concerning likely targets for preventive intervention.

The malleability and practical utility of such targets can be tested more comprehensively through microtrial methods (Howe et al., 2010) that employ elements of BTMM designs. Microtrials are randomized experiments testing the effects of relatively brief and focused environmental manipulations hypothesized to target and change specific etiologic mechanisms, but not predicted to bring about full prevention effect in distal outcomes. The study of attention bias modification by Fox et al. (2011) used such a design. Although not included in the report, the study could have tested whether baseline values of the target moderated effects on the risk mechanism.

When genes are also measured, this design also allows for tests of whether genetic moderation is in fact due to baseline target moderation, providing an even stronger test of the genetic moderation hypothesis. That is, if genetic variation is correlated with baseline variability in the hypothesized malleable target mechanism, baseline variability in the target mechanism may account for observed genetic moderation effects. Explicating genetic moderation effects in this way would require expanding the BTMM model to test whether variation in baseline target is associated with genetic variation, in effect mediating any gene-by-manipulation interaction on changes in the target. If this association is present, the baseline-by-manipulation interaction is effectively mediating a gene-by-manipulation interaction. **Figure 2** illustrates this genetically informed BTMM model.

A key assumption in both laboratory experiments and microtrials is that we have selected a valid target mechanism: that is, these designs do not provide evidence that changing the putative target mechanism will have longer-term impact on distal outcomes, only that the proximal target is malleable, and if it does carry impact, it will do so more strongly for those who need to change in that area. We can however use findings from genetically informed prevention trials to test the distal effects of putative targets, particularly if we incorporate elements of BTMM designs.

As an example, Brody et al. (2015) assessed genetic variation in DRD4 in a randomized trial of the Adults in the Making program, based in part on data suggesting that activation of the dopamine system was associated with selective attention to drug-related cues. They found that DRD4 and a family risk index together moderated the impact of the intervention on drug-related cognitions in youth. Changes in the cognitions were in turn related to changes in subsequent drug use. This model could be further extended through including both baseline levels of cognitions and information on DRD4 variation, and testing whether baseline cognitions mediate the moderating effects of DRD4 on intervention effects on subsequent change in cognition.

Baseline target moderated mediation designs can be used to test whether specific intervention targets are important, so why expand the design to include genetic variation? In particular, to further our understanding of the mechanism itself, findings that baseline target levels do mediate associations with specific genes will suggest that other mechanisms associated with those genes may also be important to explore in order to improve the next generation of prevention trials.

EXAMPLE OF FULL BTMM MODEL WITH SIMULATED DATA

We used simulation to provide an example of a full BTMM model that incorporates genetic information, and to evaluate estimation bias when the model was estimated with standard structural equation modeling methods. Using the Monte Carlo facility in MPLUS version 7.31 (Muthén and Muthén, 1998–2010), we created 1000 simulated datasets, each with 500 participants. Each dataset simulated data to match that of a clinical trial having roughly equal numbers of participants assigned to either a control or intervention condition (COND). It included scores from three indicators of a putative target (T) measured twice, once at baseline and once at post-test. It also included scores from three indicators of the study outcome (Y) also measured twice, once at post-test and once at follow-up.

For the measurement part of the model, the population model used in the simulation specified two latent variables indexing the target at the two time points, with the three specific indicators loading on their respective latent variables with equal loadings. It also specified similar latent variables indexing outcome at the two time points. Loadings for each latent variable were fixed at one for one indicator, and allowed to vary across datasets for the other two indicators. These latent variables were then used to specify two higher-order latent change variables, one for change in the target and one for change in the outcome, using a standard latent change score model (McArdle, 2009), as illustrated in **Figure 2**. Cross-time indicators were set to covary with a value of 0.20 for one pair of indicators for each of the two latent change variables, to reflect cross-time conditional dependence often found in such models.

The population model also specified a binary intervention condition variable, with proportion of intervention subjects allowed to vary across trial. Across the 1000 replications the intervention group averaged 44.6% of the total sample. We also constructed a variable to carry information about the interaction of condition with the baseline target latent variable. MPLUS uses a random effects model to allow for such interactions. We also specified a binary variable to simulate information about genetic variability in a candidate gene, such that 15% of the sample carried at least one allele associated with higher levels of the baseline mediator.

The population model specified three regressions in the structural portion of the model. Change in the outcome was regressed on post-test level of the outcome, change in the target, and condition. All three effects were set to a value of 0.25 in the population model, but allowed to vary across the different datasets. Change in the target was regressed on baseline level of the target (parameter $= -0.05$), condition (parameter $= -0.30$), and the random effect carrying information on condition by target interaction (parameter $= -0.30$). These values were chosen to reflect the situation where control and intervention conditions showed little change in the target when baseline values were low, the control group showed almost no increase in rates of change in the target regardless of baseline levels, but the intervention group showed increasing rates of change as baseline levels increased. These effects reflect the pattern of moderated mediation predicted when an intervention successfully shapes a putative target. And finally, the baseline target was regressed on the genetic variable, with a parameter of 0.30.

We used the MPLUS Monte Carlo facility to estimate and combine results from all 1000 datasets, using the model illustrated in **Figure 2**, and including correlations among cross-time indicators as well as the regression of baseline target on genetic variation (which do not appear in the Figure). We used the standard robust maximum likelihood estimator with numerical integration. Aggregated results provide information about potential bias and coverage of the model. Bias in

TABLE 1 | Estimates of bias and coverage for selected parameters from Monte Carlo simulation of data from a genetically informed baseline target moderated mediation study.

			Model parameters			Model standard errors			
			Population	Estimate	Bias (%)	Population	Estimate	Bias (%)	Coverage
Measurement model									
Target	TI	BY							
(pretest)	T1A		1	1	0.00	0	0		
	TIB		1	1.001	−0.10	0.0533	0.0522	2.06	0.93
	TIC		1	1.0022	−0.22	0.0525	0.0516	1.71	0.95
Target	T2	BY							
(post-test)	T2A		1	1	0.00	0	0		
	T2B		1	1.0002	−0.02	0.0387	0.0381	1.55	0.95
	T2C		1	1.0007	−0.07	0.0372	0.0378	−1.61	0.96
Outcome	Y2	BY							
(post-test)	Y2A		1	1	0.00	0	0		
	Y2B		1	1.0022	−0.22	0.0532	0.0518	2.63	0.94
	Y2C		1	1.0023	−0.23	0.0504	0.0514	−1.98	0.96
Outcome	Y3	BY							
(Followup)	Y3A		1	1	0.00	0	0		
	Y3B		1	1.0001	−0.01	0.0297	0.0295	0.67	0.95
	Y3C		1	1.0009	−0.09	0.0299	0.0293	2.01	0.94
Structural model									
Change in outcome	DEL23Y	ON							
	Y2		0.25	0.2549	−1.96	0.0707	0.0731	−3.39	0.95
	DEL12T		0.25	0.2502	−0.08	0.058	0.057	1.72	0.95
	COND		−0.25	−0.2517	−0.68	0.1046	0.1059	−1.24	0.96
Change in target	DEL12T	ON							
	TI		−0.05	−0.0473	5.40	0.0863	0.0838	2.90	0.95
	CONDXT1		−0.3	−0.2968	1.07	0.1074	0.1056	1.68	0.94
	COND		−0.3	−0.3066	−2.20	0.1104	0.1092	1.09	0.95
Pretest mediator on genetic variation	TI	ON							
	G		0.3	0.298	0.67	0.1299	0.1319	−1.54	0.96

the measurement model parameters (0.23% or below) and associated standard errors (2.63% or below) are very low and coverage (0.93–0.96) is excellent. Estimates for the structural model are shown in **Table 1**. Bias is also low; with one exception, bias scores for parameters are below 2.2% and for standard errors below 3.4%. The regression parameter indexing the association between the baseline mediator and change in mediator shows higher bias (5.4%), likely due to the small effect size of this parameter. Coverage is again excellent, ranging from 0.94 to 0.96. These findings indicate that the BTMM model can be specified and estimated with accuracy using standard structural equation modeling approaches.

LIMITATIONS AND CONCLUSIONS

Baseline target moderated mediation designs capitalize on random assignment to prevention condition in order to buttress causal inference concerning intervention effects on both proximal targets and distal health outcomes. However, this does not extend to tests of moderation or mediation (VanderWeele, 2015). Significant moderator effects do provide strong evidence of causal heterogeneity, but conclusions about the sources of that heterogeneity are open to confounding. For example, moderating effects of baseline family communication could be due to some other historical variable that influences baseline family communication and also acts as the true moderator of intervention impact.

Similar issues arise concerning paths from mediators to outcomes (Imai et al., 2010). However, the complex moderated mediator hypothesis, if supported, would increase plausibility of causal inference because it is more difficult to find plausible confounds that fit this pattern. For example, we might posit that an intervention could have an impact on outcomes through changing some other proximal target that influences both our putative target and subsequent outcome, but it seems less plausible that the effects on the second target would be moderated by baseline levels of the first. And as discussed earlier, we can also include a variety of design elements to further bolster plausibility of causal impact, including lagged change-to-change assessment, cross-lagged analysis, and inclusion of multiple targets to test target-specific moderated mediation.

A number of years ago Sandler et al. (1991) advocated using research on risk and protective mechanisms to identify subpopulations at risk for future emotional and behavioral problems, and developing interventions that specifically targeted those mechanisms in those groups. We see the BTMM design as a useful tool for advancing and refining this aim. For example, if we find that some families are already good at communicating with adolescents (Perrino et al., 2014), and that those families do not gain long-term benefit from a prevention program through proximal changes in their communication, we can revise future programming for that particular subgroup to focus more intensively on targets that are relevant for them, based on tests of other proximal targets.

In summary, we suggest that BTMM designs and associated statistical models hold great promise for translating studies of gene-environment dynamics into prevention science. They also provide a means of testing how and when specific proximal targets of preventive intervention will have maximal impact on distal health outcome, and as a result can guide refinement of next generation prevention trials. And, given current standards for measuring both targets and outcomes at baseline as well as at post-test and follow-up, they can be easily implemented within current prevention trial designs with little or no extra cost.

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A quantitative epigenetic approach for the assessment of cigarette consumption

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Smoking is the largest preventable cause of morbidity and mortality in the world. Despite the development of numerous preventive and treatment interventions, the rate of daily smoking in the United States is still approximately 22%. Effective psychosocial interventions and pharmacologic agents exist for the prevention and treatment of smoking. Unfortunately, both approaches are hindered by our inability to accurately quantify amount of cigarette consumption from the point of initial experimentation to the point of total dependency. Recently, we and others have demonstrated that smoking is associated with genome-wide changes in DNA methylation. However, whether this advance in basic science can be employed as a reliable assay that is useful for clinical diagnosis and treatment has not been shown. In this communication, we determine the sensitivity and specificity of five of the most consistently replicated CpG loci with respect to smoking status using data from a publically available dataset. We show that methylation status at a CpG locus in the aryl hydrocarbon receptor repressor, cg05575921, is both sensitive and specific for smoking status in adults with a receiver operated curve characteristic area under the curve of 0.99. Given recent demonstrations that methylation at this locus reflects both intensity of smoking and the degree of smoking cessation, we conclude that a methylation-based diagnostic at this locus could have a prominent role in understanding the impact of new products, such as e-cigarettes on initiation of cigarette smoking among adolescents, while improving the prevention and treatment of smoking, and smoking related disorders.

Keywords: DNA methylation, epigenetics, aryl hydrocarbon receptor repressor, cg05575921, diagnostics, smoking, e-cigarettes

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Introduction

Smoking is the largest preventable cause of morbidity and mortality in the United States. Each year, nearly 1/2 million Americans die secondary to the effects of smoking (Centers for Disease Control and Prevention, 2008). Beyond the personal toll, smoking has an enormous financial impact on the United States. Each year, the U.S. spends nearly $100 billion on the treatment of smoking-related illnesses and suffers an additional $100 billion in lost wages (Centers for Disease Control and Prevention, 2008).

In response to this public health crisis, state and federal governments have implemented a series of policy measures and supported the implementation of preventive interventions by public health workers. In addition, large Pharma has collaborated with academia to develop effective medications, such as bupropion and varenicline for smoking cessation (Mills et al., 2012). Despite these efforts, 22% of all U.S. adults reported daily smoking in 2010 (Centers for Disease Control and Prevention, 2011).

Surprisingly, one of the largest barriers to developing more effective smoking prevention and cessation interventions has been our relative inability to objectively quantify tobacco consumption. Currently, there are three principal methods for determining tobacco consumption. The first is self-report. In general population samples, self-report is an adequate measure of tobacco consumption. However, in high risk populations and in adolescents, self-report is often unreliable (Fendrich et al., 2005; Jarvis et al., 2008; Gorber et al., 2009). This is especially true in higher risk clinical settings, such as pregnancy, where patients are sometimes reluctant to confide to physicians their inability to quit (Shipton et al., 2009; Dietz et al., 2011).

In attempts to supplement self-report, objective measures of tobacco consumption, such as serum or salivary cotinine or exhaled carbon monoxide levels, are sometimes used. Unfortunately, each of these approaches for determining smoking status has its limitations (Florescu et al., 2009). While easy to perform, exhaled carbon monoxide levels are only useful for detecting smoking within 3–4 h of the last cigarette (Jatlow et al., 2008; Florescu et al., 2009). Serum and salivary cotinine levels are more sensitive, generally detecting use with 48 hours, but are usually determined using more difficult to perform enzyme linked immunoassays (ELISA; Jatlow et al., 2008). These relatively narrow time windows for detection limit the usefulness of these approaches in detecting nascent smoking among adolescents during the critical smoking initiation period, or for "chippers," i.e., light and intermittent non-daily smokers that use cigarettes only in specific situations such as bars or with their first cup of coffee in the morning (Levinson et al., 2007).

Over the past several years, the limitations of cotinine based assays of smoking have made more apparent by the introduction of e-cigarettes. These devices, which vaporize a solution of propylene glycol that contains nicotine, are gaining popularity use among adolescents, with prevalence data showing that use at least doubled in the U.S. and Britain every year from 1% in 2009 to 2% in 2010, and 6–7% in 2012 (Pepper et al., 2013; Centers for Disease Control and Prevention, 2014). Although perceived by teens as being healthier than cigarettes, many e-cigarette users also smoke cigarettes, and there is considerable concern from public health experts that these devices will further increase teen smoking (Grana et al., 2014; Wills et al., 2015). Since use of these e-cigarettes, nicotine replacement agents, such as the "patch," and non-smoked forms of tobacco consumption also results in positive serum and salivary cotinine results, the usefulness of cotinine determinations in differentiating between their use and surreptitious cigarette smoking and guiding smoking cessation treatment is relatively limited. Hence, there is urgent need for new measures for the detection of cigarette consumption.

Recently developed epigenetic approaches to determine smoking status may provide the necessary tools to bridge the chasm in our ability to detect and quantitate cigarette consumption. Beginning in the first decade of this millennium, we and others demonstrated gene specific changes in DNA methylation in response to smoking (Philibert et al., 2008; Breton et al., 2009; Launay et al., 2009). When the first truly genome-wide platform for measuring smoking consumption was developed (Illumina HumanMethylation450 BeadChip), we used it to show that demethylation at a CpG residue interrogated by probe cg05575921 in the aryl hydrocarbon receptor is a sensitive and highly specific indicator of cigarette consumption (Monick et al., 2012). Since that time, numerous independent studies using this chip have confirmed these findings in DNA from newborns, adolescents, and adults (see **Table 1**; Joubert et al., 2012; Philibert et al., 2012, 2013; Shenker et al., 2012; Zeilinger et al., 2013; Besingi and Johansson, 2014; Dogan et al., 2014; Elliott et al., 2014; Harlid et al., 2014; Tsaprouni et al., 2014; Guida et al., 2015). In addition, three groups have shown that smoking induced methylation changes can revert as a function of smoking cessation and that cg05575921 is the most sensitive residue in the genome in response to smoking cessation (Zeilinger et al., 2013; Harlid et al., 2014; Guida et al., 2015). Finally, we have shown that the effects of smoking on DNA methylation are unique to smoking and are not affected by alcohol consumption, thus allowing smoking and alcohol consumption status to be assessed simultaneously from the same dataset (Philibert et al., 2014a). Taken together, these studies indicate that DNA methylation assessments hold considerable promise as a tool for supplementing self-report information in smoking prevention and smoking cessation efforts. The question is as to how they will be integrated into our current prevention and treatment framework.

For now, the studies listed in **Table 1** indicate a potential for DNA methylation to be used as an independent method to unequivocally establish the presence of smoking. This capability may be potentially useful under certain circumstances. For example, it is well established that smoking is a modifiable

TABLE 1 | Results of replication attempts of the original findings by Monick et al. (2012) with respect to methylation status at AHRR probe cg05575921 using independent populations.

Reference	Rank (of 485557 probes)	FDR *P*-values	Population
Philibert et al. (2012)	1st	3×10^{-7}	Adolescents
Joubert et al. (2012)	1st	8×10^{-33}	Newborns
Shenker et al. (2012)	1st	2×10^{-15}	Adults
Philibert et al. (2013)	1st	2×10^{-3}	Young adults
Zeilinger et al. (2013)	1st	3×10^{-182}	Adults
Dogan et al. (2014)	2nd	6×10^{-19}	Adults
Besingi and Johansson (2014)	1st	7×10^{-70}	Adults
Elliott et al. (2014)	1st	6×10^{-59}	Adults
Tsaprouni et al. (2014)	1st	9×10^{-69}	Adults
Harlid et al. (2014)	2nd	2×10^{-2}	Adults
Guida et al. (2015)	1st	1×10^{-106}	Adults

risk factor for certain high risk medical procedures with many physicians refusing to operate unless the patient has quit smoking (Peters et al., 2004). Or, in efforts to promote a healthier workforce, prominent governmental bodies such as the World Health Organization (WHO) as well as many private employers are refusing to employ smokers (Cage, 2005). By taking advantage of the inherent stability of methylation signatures over short periods of time, the potential surety of detection afforded by these methylation technologies provides a framework around which the appropriate incentives can be placed to improve medical outcomes and decrease overall healthcare costs.

However, in order for that vision be realized, the current genome wide approaches need to be reduced to a potentially clinical format. In hopes of accomplishing this, using the information generated in these studies, our academic/corporate consortium devised an easy to use quantitative PCR assay of cg05575921 methylation status referred to as Smoke Signature (Dogan et al., 2014). Nevertheless, the question remains as to whether determination of methylation at this locus or any other of the loci that were commonly identified in the prior studies are solely capable of determining smoking status.

As a first step in answering this question, in this study, we use publically available methylation data from a recently completed study and standard analytic approaches to test single and multiple locus approaches to the determination of smoking consumption.

Materials and Methods

The data used in the study are derived from subjects who participated in a previously described National Institutes of Health study that examined the effects of alcohol on DNA methylation (R43AA022041; Philibert et al., 2014a). All protocols and procedures used in this study were approved by the University of Iowa Institutional Review Board.

In brief, the drinking participants (drinkers) were recruited from either local alcohol treatment centers or the University of Iowa Hospitals and Clinics for the treatment of alcohol dependence. Participants were approached after they had detoxified from alcohol intake (between 3–7 days after the last drink). The inclusion criteria for the study specified good overall health and the absence of active substance use outside of alcohol or tobacco. Furthermore, participants could not be taking any medication hypothesized to affect DNA methylation (such as valproic acid). The controls (non-drinkers) were recruited from the University of Iowa community and were required to be abstinent from alcohol and all other forms of substance use with the exception of tobacco. All participants reported the number of cigarettes smoked per day over the past month and past year.

After consent was obtained, all participants were interviewed with a modified version of the Semi Structured Assessment for the Genetics of Alcoholism, Version 2 (SSAGA-II) by a trained research assistant (Bucholz et al., 1994). The SSAGA-II is a publically available standardized interview that demographic and modules for each of the major behavioral disorders with particular emphasis on the substance use disorders (see Appendix 1). This information was supplemented by a questionnaire that assessed consumption of substances over the past day, past week, past month, past 6 months, and past year (see Appendix 2). They were then phlebotomized to provide the biomaterial for the current study. Serum samples were obtained using standard serum separator tubes and stored at −80°C until analyzed. Mononuclear cell pellets were obtained via gradient centrifugation of whole blood through Ficoll as previously described (Philibert et al., 2012). DNA was then prepared from these samples using a QiaAMP DNA (Qiagen, Germany) according to the manufacturer's instructions.

We defined smokers as those individuals who reported the recent use of cigarettes or other forms of combustible tobacco while we defined those who did not use any type of combusted tobacco or cannabis as non-smokers. In order to confirm self-reported smoking status, serum cotinine and hydroxy-tetrahydro-cannabinol (hydroxy-THC) levels were assessed using immuno ELISA supplied by Abnova (Taiwan) according to manufacturer's directions. Data from one participant whose serum assessments were not consistent with self-report were excluded from further analysis in the study. Because the serum cotinine levels are highly dependent as to the time of the last cigarette and the two of the facilities where we recruited subjects did not allow free access to cigarettes at all time, we used serum cotinine levels as only as an indicator of smoking status and not as an indicator of total cigarette consumption.

The methylation data for the five loci described in the current study were extracted from the previously conducted genome-wide methylation assessments which are publicly available (GEO accession number GSE57853). These DNA methylation assessments were conducted by the University of Minnesota Genome Center using the Illumina HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA; Philibert et al., 2012, 2013). The resulting data were inspected for complete bisulfite conversion. Then average β-values (the ratio of the methylated probe fluorescence intensity to the sum of the methylated and unmethylated probe fluorescence intensities) were determined using the GenomeStudio® suite of programs. These values were then cleaned using a Perl-based algorithm to remove unreliable data points before deposition into the Gene Expression Omnibus (GEO) website (Dogan et al., 2014).

Clinical and demographic data were then analyzed using JMP version 10 (SAS Institute, Cary, NC, USA, software company) using the tests indicated in the text. The Receiver Operator Characteristic analyses were also conducted using this package.

Results

In the previous study of the effects of alcohol consumption on DNA methylation, we used data from a total of 66 participants. For the purposes of the current study, we excluded the data from five of those participants. The first was excluded because his substance use self-report of abstinence was not consistent with our serum ELISA assessments. The second and third were excluded because while they were not current smokers, they were both cigarette smokers in the past 10 years and were currently smoking cannabis-which is commonly mixed with tobacco to

improve pyrolysis. The fourth and fifth were excluded from the primary analyses because they used chew or snuff which precluded serum verification of smoke free status.

The demographic characteristics of the remaining 61 participants whose data are included in the main analyses are given in **Table 2**. Overall, the middle-aged participants were mostly male and white. Only two of the smokers did not have a history of recent alcohol consumption. All of the participants who reported daily smoking had detectable levels of cotinine in their serum (average 99 ± 42 ng/ml). Please note that because all of the drinkers were ascertained in smoke-free facilities several days after admission when they had detoxified from alcohol intake, the levels of cotinine observed in the current study are probably not representative of daily cigarette consumption prior to admission. Nine of the smokers had positive tests for cannabis consumption.

The loci selected for this study are the five most commonly replicated loci and the only five loci that are consistently demethylated in both European and African American populations (Dogan et al., in submission). As a first step of our analyses, we conducted ANOVA analysis of the case and control data using methylation at these loci as the dependent variable (see **Figure 1**). Overall, the model that included cg05575921 provided the best fit and the largest arithmetic differences (21%) between cases and controls (adjusted $R^2 = 0.66$). The results from the three loci on Chromosome 2, cg01940273, cg21566642, and cg05951221 provided the next best fits with adjusted R^2-values of 0.55, 0.50, and 0.44, respectively. However, the differences in the means of the Chromosome 2 loci were much more modest, ranging from approximately 8–10%. Finally, the model that used data only from cg23576855 was the worst fit with an adjusted R^2 of 0.34. Consistent with recent studies showing that methylation in these arrays and at this locus in particular is often affected by local genotype (Shenker et al., 2012; Philibert et al., 2014b) visual inspection of the data showed strong evidence of GxMeth effects with respect to smoking (data not shown).

Although our smoking subjects did not exclusively smoke cigarettes, the main mode of tobacco consumption for our subjects was cigarette smoking. Therefore, we next analyzed the relation between DNA methylation at each locus with self-reported average smoking in the past month and past year using a linear bivariate fit model. In general, methylation at cg05575921 produced the best fit, with the three Chromosome 2 probes producing intermediate levels of fit, and cg23576855 produced the worst fit (see **Table 3**). In attempts to improve the goodness of fit of the model, we then tested whether log transformation of either absolute methylation or number of cigarettes consumed could improve the fit of the models. Unfortunately, no consistent improvements in model effects were obtained.

As the final step of our analyses, we used data from all five loci alone and in combination with one another, in an attempt to determine whether data from a single marker or multiple markers is optimal for the discrimination of smokers from non-smokers. When only single markers were considered, receiver operating characteristic model (ROC) analyses of the data showed that cg05575921 provided the best discrimination with area under the curve (AUC) of 0.99 (**Table 4**). Review of the logistic fit curve for cg05575921 with respect to smoking status shows excellent sensitivity for these smokers at all ranges of specificity (**Figure 1**). DNA methylation at the other four loci, in particular, cg01940273 were slightly less discriminative with the use of a two marker

TABLE 2 | Clinical and demographic characteristics of subjects included in main analysis.

	Non-smokers	Smokers
N	35	26
Age	47 ± 8	46 ± 7
Gender		
Male	28	18
Female	7	8
Ethnicity		
White	33	24
African American	1	2
Hispanic	1	0
Daily cigarette consumption in the past month	0	20 ± 8
Substance use status		
Alcohol	4	24
Positive Cotinine	0	26
Positive Hydroxy THC	0	9
Average methylation (% β)		
cg05575921	90.3 ± 1.9	68.8 ± 11.6
cg01940273	59.8 ± 4.4	49.9 ± 4.6
cg21566642	46.2 ± 4.8	34.2 ± 7.0
cg05951221	39.5 ± 4.2	31.1 ± 5.2
cg23576855	67.0 ± 13.1	49.2 ± 11.1

TABLE 3 | The relationship of DNA methylation to average cigarette consumption in the past month and past year.

	Adjusted R^2 of linear fit model for average cigarette consumption	
CpG probe	**Past month**	**Past year**
cg05575921	0.64	0.63
cg01940273	0.57	0.56
cg21566642	0.46	0.45
cg05951221	0.47	0.45
cg23576855	0.30	0.32

The adjusted R^2 is based on a linear fit model.

TABLE 4 | Summary of ROC area under the curve (AUC) analyses for single and two marker sets.

		When combined with methylation from another locus		
	Alone	**cg21566642**	**cg05951221**	**cg23576855**
cg05575921	0.990			
cg01940273	0.940	0.939	0.946	0.947
cg21566642	0.905		0.918	0.923
cg05951221	0.903			0.919
cg23576855	0.860			

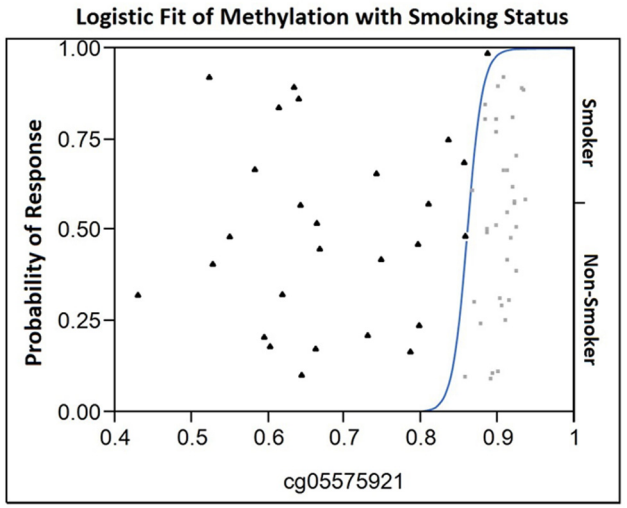

FIGURE 1 | The Logistic Fit plot of the relationship between methylation at cg05575921 and smoking status. Observed cg05575921 methylation for each 61 subjects in the study is shown on the *X*-axis (smokers are represented by a filled triangle, non-smokers by a light gray box) with their position on the *Y*-axis not having meaning. In contrast, the position on the *Y*-axis with respect to the fitted curve indicates the probability for each response (either smoker or non-smoker) for a given value on the *X*-axis. In essence, for those subjects with a methylation level less than 0.82 have essentially no chance of being in the non-smoker group. At a methylation level of approximately 0.85, the probability of being a smoker is approximately 0.75 and the probability of being a non-smoker is 0.25. Then, the probability of being non-smoker then rises as a function of increasing methylation becoming virtually certain at levels of 0.9 or greater.

set consisting of the Chromosome 2 probe cg01940273 and the AHRR probe cg23576855 resulting in an AUC of 0.947.

Discussion

In this limited, but well characterized set of participants, we show that DNA methylation status at cg05575921, and to a lesser extent at three Chromosome 2 loci, can be used to accurately quantify the amount of smoking. Important limitations of the current study include sample size and the limited diversity in the subject pool.

To a large extent, the strength of the findings is in large part due to the careful selection and characterization of the participants. In our experience with several large cohorts of subjects from longitudinal studies, we often find that participants who deny ever smoking cigarettes often have markedly elevated levels of cotinine in their serum and have medical illnesses, such as chronic obstructive lung disease (COPD), that are generally found in association with smoking. Review of the literature suggests that our experience is not unique. In 2007, Gorber et al. (2009) conducted a meta-analysis of 67 studies of the relationship between self-reported smoking status and smoking status as determined by serum, urine, or salivary cotinine levels. They found trends of underestimation of the true rate of smoking when smoking status is based only on self-report depending on the population studied. These findings confirmed the earlier results from Fendrich et al. (2005) who found that the sensitivity of self-report in a large ($n = 627$) cohort from an epidemiological study was less than 90%, even after generous compensation for passive exposure. In order to minimize the likelihood that our controls smoked, we recruited our controls from the employee pool of our hospital complex which forbids smoking. Even so, it is notable that one of the participants who reported no smoking history had a positive test for cannabis consumption. Hence, the level of methylation at cg05575921 observed in the non-smoking participants in our study (β% of 90.3 ± 1.9) is probably an accurate reflection of adult methylation values in the complete absence of smoking and highlights the need for intense scrutiny and serum confirmation of non-smoking controls. In this regard, the substance use status participants from almost all of the studies listed in **Table 1** were not biochemically verified. Hence, it is likely that small numbers of smokers were misclassified and that as a result, the average β-values for the non-smoking groups were underestimated.

Even though several of the loci showed considerable promise for possible clinical translation, it is important to realize that the ROC AUC calculations were conducted using data from methylation microarrays. This hybridization-based approach is performed under meticulous conditions and takes several days to complete. After the assessment is complete, sophisticated computational processing is then required to extract the normalized β-values. It is unlikely that this assessment approach can be adapted to the point of care (POC) or hospital-based pathology lab practice.

In contrast, quantitative PCR (qPCR) techniques are becoming increasingly common in clinical settings (Lorincz, 2014). At least one epigenetic diagnostic is already FDA approved, and it is likely that several others will gain approval in the near future (Heichman, 2014; Lorincz, 2014). Like all qPCR assessments, the power of these tests to distinguish groups from one another is dependent on the variability of the assay itself, and the absolute difference between the two groups. In normal practice, inter-assay variability of approximate 1–2% is routinely observed for most qPCR assays. Because the average difference at cg05575921 between adult smoker and non-smokers is approximately 21%, while the absolute difference at the three Chromosome 2 loci is only 8–10%, it is readily apparent that the AHRR site is a better choice for clinical systems. This is why our initial assay was targeted at this locus (Dogan et al., 2014). However, with the appropriate amount of effort, it is still may be feasible to pursue clinical tests for adults based on the other loci. Unfortunately, this is not the case for any diagnostic targeted at adolescents because the magnitude of change at the Chromosome 2 loci in nascent smokers is only on the order of 1–2% (Philibert et al., 2012, 2013). In contrast, the change at cg05575921 is much more robust (5–10%) and is a much more suitable locus for detection of adolescent smokers.

This methylation-based assessment technique could be particularly valuable for understanding the relation between the use of e-cigarettes and cigarette smoking. The changes in AHRR methylation are not secondary to nicotine consumption itself. Rather, AHRR methylation is an exquisite indicator of exposure

to the dioxins and polyaromatic hydrocarbons (PAH) found in cigarette smoke. Indeed, in the current study, the two subjects excluded from our study secondary to the use of "chew" had the exact same methylation at cg05575921 (90.3 and 90.9 β%) as our non-smoking controls (β% of 90.3 ± 1.9) confirming prior findings by Besingi and Johansson (2014) that nicotine ingestion itself has no effect on AHRR methylation. Although it is true that the heat filament induced vaporization of the propylene glycol solution also produces small amounts of potentially concerning byproducts, the extent of these pollutants, in particular dioxins and PAH, appears to be relatively small (McAuley et al., 2012; Schober et al., 2014). To date, the most incriminating study of dioxins or PAH in e-cigarette aerosols showed a total of 96 ng of PAH and no dioxins being produced from the pyrolysis of an entire e-cigarette cartridge (equivalent to the puffs of about 15 cigarettes; Laugesen, 2008). For the sake of reference, this corresponds to approximately 11 pg/ml in the typical 35 ml puff-, which is about the 30 times the PAH content in urban or rural air (Li et al., 2005; Primbs et al., 2008). Because the average human breathes 8–12 times per minute with an average tidal volume of 500 ml, smoking "e-cigarettes" essentially doubles the amount of PAH inhaled *only* while smoking the e-cigarette. In contrast, the PAH just the mainstream smoke of the equivalent number of cigarettes is between 15000 and 24000 ng of PAH (Ding et al., 2005). Hence, those who smoke e-cigarettes only should not have an appreciable change at cg05575921 but have positive cotinine levels while those who are smoking real cigarettes will have both changes at cg05575921 and a positive cotinine level. Therefore, the amount of incorporation of DNA methylation assessments into research protocols could provide valuable biological information to longitudinal studies of the relationship of e-cigarette use to subsequent cigarette smoking.

An additional boon to potential clinical translation is the fact that methylation in DNA from blood is closely correlated to that obtained from saliva. In fact, one recent study that provided analyses of paired samples from the same person demonstrated a correlation of 0.90 of cg05575921 methylation in DNA drive from blood and saliva (Smith et al., 2015). Unfortunately, unlike blood, the principal cell components found in saliva differ significantly with respect to their methylation set point at this locus. Therefore, techniques that can compensate for cellular heterogeneity will be required before saliva DNA methylation approaches can be used alongside blood-based approaches in the assessment of smoking status. Our group is currently working on one such technique.

Somewhat ironically, these methylation assessments may increase our ability to improve self-report measures. It goes without saying that bad questions asked poorly illicit are likely to elicit unreliable answers. A shortcoming of prior assessments of self-report reliability with respect to adolescent smoking was that the methods to assess reliability themselves seldom performed objective testing and when they did they only tested cotinine levels (Gorber et al., 2009). The current findings suggest that the addition of methylation assessments may increase our confidence in identifying true positives and true negatives, resulting in an improved mechanism through which to evaluate methods of obtaining substance use histories.

A critical question not addressed in this manuscript is whether changes in DNA methylation at cg05575921 can be used as a marker of smoking cessation. Already, three independent studies have shown that this is also the locus that shows the most significant change in response to cessation of smoking. There are two principal challenges to the use of methylation status at this AHRR locus in this regard. First, since the average methylation for heavy smokers seems to vary widely, any assessment of tobacco cessation will have to take into account the initial methylation status of the patient in question. Second, the half-life for decay of the smoking induced changes at this locus will have to be much better characterized. All three of the studies that showed the primacy of cg05575921 remethylation in response to smoking cessation were based solely on self-report data. Since the self-reports of "former smokers" can be unreliable as to the extent and timeframe of smoking cessation (Attebring et al., 2001), and the true "set point" of cg05575921 is still being refined, examination of this phenomenon in large, well-characterized samples (i.e., frequent biochemical validation) will be required before the viability of this approach for assessing smoking cessation can be considered. Still, given the positive response of smokers to biofeedback information from exhaled carbon monoxide measurements, the possibility that patients could gain enhanced motivation to quit smoking by seeing methylation changes at loci, such as F2RL3, which is implicated in heart disease risk (Breitling et al., 2012; Zhang et al., 2014), as a function of smoking cessation suggests that this possibility deserves further exploration. Currently, in efforts funded by the National Institute of Drug Abuse, our consortium is pursuing a small pilot study to explore the feasibility of this approach.

In summary, using data from well-characterized, biochemically verified participants, we show that DNA methylation assessments, particularly at cg05575921, are very sensitive and specific indicators of smoking status in adults. We suggest that additional study of large, well characterized, biochemically confirmed, epidemiological representative populations are the next logical step in the translation of this approach into routine clinical, research, and commercial usage.

Acknowledgments

The use of DNA methylation to assess alcohol use status is covered by pending property claims. The use of DNA methylation to assess smoking status is covered by U.S. patent 8,637,652 and other pending claims. RP is a potential royalty recipient on those intellectual right claims. Both TO and RP are officers and stockholders of Behavioral Diagnostics (www.bdmethylation.com).

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpsyg.2015.00656/abstract

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Conflict of Interest Statement: Dr. Robert Philibert is the majority owner and Chief Scientific Officer of Behavioral Diagnostics. He has pending and granted intellectual property rights for the use of DNA methylation techniques to quantify substance use consumption. Dr. Terry Osborn is the Chief Excecutive Officer and partial owner of Behavioral Diagnostics.

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REVIEW
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Impulsive delayed reward discounting as a genetically-influenced target for drug abuse prevention: a critical evaluation

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This review evaluates the viability of delayed reward discounting (DRD), an index of how much an individual devalues a future reward based on its delay in time, for genetically-informed drug abuse prevention. A review of the literature suggests that impulsive DRD is robustly associated with drug addiction and meets most of the criteria for being an endophenotype, albeit with mixed findings for specific molecular genetic influences. Several modes of experimental manipulation have been demonstrated to reduce DRD acutely. These include behavioral strategies, such as mindfulness, reward bundling, and episodic future thinking; pharmacological interventions, including noradrenergic agonists, adrenergic agonists, and multiple monoamine agonists; and neuromodulatory interventions, such as transcranial magnetic stimulation and transcranial direct current stimulation. However, the generalization of these interventions to positive clinical outcomes remains unclear and no studies to date have examined interventions on DRD in the context of prevention. Collectively, these findings suggest it would be premature to target DRD for genetically-informed prevention. Indeed, given the evidence of environmental contributions to impulsive DRD, whether genetically-informed secondary prevention would ever be warranted is debatable. Progress in identifying polymorphisms associated with DRD profiles could further clarify the underlying biological systems for pharmacological and neuromodulatory interventions, and, as a qualitatively different risk factor from existing prevention programs, impulsive DRD is worthy of investigation at a more general level as a novel and promising drug abuse prevention target.

Keywords: substance use disorders, drug abuse, addiction, behavior economics, delayed reward discounting, behavioral economics, intertemporal choice

Introduction

Excessive use of addictive drugs is both widespread and onerous, contributing to to approximately 22% of deaths and costing more than $500 billion annually in the United States (Mokdad et al., 2004; Uhl and Grow, 2004). A high priority for reducing the burden of addictive disorders is to translate knowledge of the underlying risk factors for addiction into prevention and early intervention approaches. Numerous factors influence the probability of initiation and progression of drug use,

but one well established domain is genetic variation, which is estimated to contribute approximately half of the liability for developing drug addiction (Goldman et al., 2005; Agrawal and Lynskey, 2008). From a theoretical perspective, aligning prevention efforts to address genetic risks has very high potential, as it would focus on important etiological variables that are not currently considered from a prevention perspective and would seek to assist individuals who are constitutionally at elevated risk. It would be a form of personalized medicine, but at the level of prevention. Ideally, genetically-informed prevention programming would go one step further and would target the specific ways that genetic variation give rise to drug abuse risk, the biopsychosocial mechanisms of risk. By permitting very early risk identification and the delivery of maximally relevant prevention programming, prevention strategies that are specifically tailored to genetically-influenced risk mechanisms would have the potential to have a major impact.

At a behavioral level, an increasingly well-established risk factor for drug abuse is impulsive decision making, specifically, the propensity to select an immediate reward at the expense of greater future rewards. This form of impulsivity is typically referred to as delayed reward discounting (DRD) or capacity to delay gratification, and has also been increasingly linked to genetic influences. The link to genetics in turn suggests that impulsive DRD may be a viable candidate for genetically-informed prevention. In practice, what this means is that individuals with genetic profiles associated with more impulsive DRD would pre-emptively receive programming to reduce preferences for immediate gratification and, ultimately, to reduce the probability of subsequent drug abuse. This would be a radically different strategy from current prevention efforts and could be very powerful, both for preventing drug abuse and a number of adverse health outcomes. However, it is also lofty prospect that is highly contingent on a number of relationships being empirically robust.

The goal of the current review is to concretely evaluate the existing literature on the prospects of DRD as a genetically-informed prevention target. The review has four goals: (1) to introduce DRD as a behavioral characteristic and review its association with drug abuse; (2) to review the evidence suggesting DRD is an endophenotype (i.e., a genetically-influenced mechanism of risk for addictive disorders); (3) to review candidate intervention approaches for reducing impulsive DRD; (4) to critique the extent to which the preceding sections "connect the dots" to make an compelling argument for such an approach.

Methodology

To conduct our review, we examined the published literature using the Public Library of Medicine (PubMed) and PsychINFO databases. Specifically, we examined individual empirical articles and reviews that addressed DRD in the context of drug abuse, behavioral genetics, and manipulations or interventions that reduce impulsive DRD. The review included studies on DRD in both humans and non-human animals, but, given that DRD is generally independent of other measures of impulsivity (e.g., MacKillop et al., 2014), we did not include studies of other domains. Articles in the two major domains of the study (genetic influences on DRD and strategies for reducing DRD) were critiqued in detail and the accompanying cited works were used to identify other potentially relevant studies. However, a specific search protocol using a discrete set of search terms was not implemented, meaning that this article is more appropriately considered a critical review of the literature, but not a formal systematic review (e.g., Khan et al., 2003).

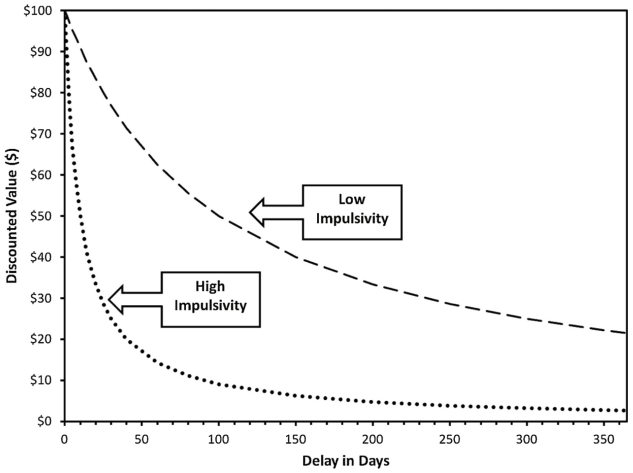

FIGURE 1 | Prototypic hyperbolic delayed reward discounting curves reflecting the discounted subjective value of $100 delayed from 1 day to 1 year The curves reflect the points at which the smaller immediate reward is equal in value to the $100 delayed reward. For example, at a delay of 100 days, $100 has lost ∼50% of its nominal value for the low impulsivity profile and ∼90% of its nominal value for the high impulsivity profile. Figure from MacKillop (2013).

Delayed Reward Discounting and Drug Abuse

Delayed reward discounting is typically assessed using decision-making tasks posing choices between a smaller monetary reward that is immediately available and a larger delayed reward after an intervening delay. By varying the reward amounts and the delay length, an overall characterization of temporal discounting rates can be generated. **Figure 1** provides illustrative discounting temporal discounting curves for $100 available in the future versus smaller amounts available in the present. Quantitative indices of discounting are typically generated either using non-linear regression to derive an individual's temporal discounting function (i.e., *k*) or generating the area under the curve (AUC; Myerson et al., 2001). The *k* index reflects the slope of the hyperbolic discounting function and AUC reflects the overall volume of the discounting curve. The two are strongly inversely correlated; steeper discounting curves have high *k* values and low AUCs. Both indices have their own advantages and disadvantages, with the primary difference being that *k* makes implicit assumptions about the hyperbolic form of the discounting function, whereas AUC is theory-free and does not make assumptions about the specific form of the curve (Myerson et al.,

2001). Although often administered for hypothetical outcomes, a number of studies suggest that individuals respond similarly on versions of the task in which they are provided with an actual monetary reward based on their responses (Johnson and Bickel, 2002; Madden et al., 2003; Bickel et al., 2009; Lawyer et al., 2011). Furthermore, studies have demonstrated robust test-retest reliability, with comparable stability to personality traits (Beck and Triplett, 2009; Kirby, 2009; Odum, 2011). Money is the most commonly used commodity and has a number of advantages (e.g., generality of relevance, meaningfulness of discrete units), but other commodities, including addictive drugs, can be assessed using DRD paradigms. Indeed, some of the earliest work in this area used the now famous "marshmallow test" in which children choose between one marshmallow immediately available after the experimenter leaves the room or two marshmallows if they wait for the experimenter to return (Metcalfe and Mischel, 1999).

Individuals who strongly prefer immediate over delayed rewards of larger value are said to exhibit impulsive discounting of delayed rewards. Impulsive DRD is associated with earlier age of addiction onset (Dom et al., 2006), dependence on multiple classes of drugs (e.g., tobacco, alcohol, cocaine; MacKillop et al., 2011), and treatment response (MacKillop and Kahler, 2009; Sheffer et al., 2014). Furthermore, a meta-analysis synthesized previous literature on DRD in relation to addictive behavior by comparing levels of DRD between criterion (addicted) groups and control groups (MacKillop et al., 2011), finding consistent evidence of significantly more impulsive DRD in criterion groups, with a medium effect size across studies ($d = 0.58$).

Although debate has arisen regarding the extent to which DRD is a cause or consequence of addiction (or whether they are both influenced by a third variable), there is increasing evidence that DRD preferences at least partially predates the development of addiction. Two retrospective studies have identified that more impulsive DRD predicts earlier onset of alcohol use (Kollins, 2003) and alcohol use disorder symptoms (Dom et al., 2006). Several subsequent studies have found a link between more impulsive DRD in adolescents and increased substance use and/or misuse over time (Audrain-McGovern et al., 2009; Fernie et al., 2013; Khurana et al., 2013; Kim-Spoon et al., 2014). However, one recent study did not find a consistent connection between DRD and subsequent substance use (Isen et al., 2014). Notably, the null results in this study may be partially attributable to their utilization of an externalizing latent factor that included a broader spectrum of externalizing behaviors than simply drug use and misuse (e.g., disinhibited, delinquent, and aggressive behavior). In sum, DRD is a well-validated behavioral measure of impulsivity, is consistently associated with addictive behavior, and is an etiological risk factor, predating alcohol and tobacco use and misuse.

Delayed Reward Discounting as a Drug Abuse Endophenotype

Although approximately ∼50% of all variance in addictive disorders is genetic risk (Goldman et al., 2005; Agrawal and Lynskey, 2008), little variance has been consistently accounted for by molecular genetic studies. In fact, candidate gene studies (assessing associations with a small number of variants in a limited number of genes) and genome-wide association studies (assessing associations with hundreds of thousands of variants across the genome) have both identified variants which are inconsistently replicated and exhibit small effect sizes (Goldman et al., 2005; Treutlein and Rietschel, 2011). This gap between high levels of heritability and specific variants of inconsistent and small effects is referred to as the "missing heritability problem" (Turkheimer, 2011). Several potential factors contribute to this issue, but perhaps two are most notable: (1) addictive disorders are highly polythetic (i.e., hundreds of combinations of symptoms can produce the same diagnosis); and (2) addictive disorders are "too far" from the genes, meaning that the proximal consequences of genetic variation may be only distantly related to the proximal risk factors for drug abuse. As a result of these obstacles, an endophenotype approach has been proposed, shifting the focus to narrower phenotypes that are putatively determined by a more limited number of genes and are more specifically associated with the disorder of focus. Endophenotypes are also intended to be mechanistically informative about the nature of genetic influences. Given both links to genetics and mechanisms of risk, endophenotypes are the natural intervention targets in the context of genetically-informed prevention.

Importantly, a number of criteria have been increasingly accepted as defining an endophenotype. These comprise evidence of the following: (1) association with the illness, meaning a link with the condition of interest; (2) heritability, meaning evidence that the characteristic is influenced by genetics; (3) state independence, meaning the characteristic is present when the disease is not (and is not simply a symptom of the condition); (4) present in non-affected family members at higher rates than the general population, further indicating its genetic basis; and (5) co-segregation with the psychiatric illness in families, further indicating association (Gottesman and Gould, 2003).

For DRD, the first of these criteria was addressed above, in the links between the behavioral characteristic of DRD and drug abuse. Shifting to the heritability of DRD, there is robust evidence from animal and human studies. Animal studies are particularly useful for assessing heritability of traits because they allow researchers to control all aspects of the environment. The reduction in environmental variability enables isolation of the effects of genetic variability. In animal studies, researchers typically compare behaviors across inbred strains that are isogenic (i.e., entirely or nearly genetically identical; Falconer et al., 1996). In the first rodent study of DRD heritability, approximately 16% of variability in DRD rates was attributable to between-strain differences in mice (Isles et al., 2004). Studies of Lewis and Fischer rodents reared in identical environments also identified systematic differences in discounting across strains that are attributable to genetic differences (Anderson and Woolverton, 2005; Madden et al., 2008; Stein et al., 2012). Finally, in a recent study, the estimated heritability across eight strains was between 43 and 66% (Richards et al., 2013). Overall, these studies largely found robust differences in DRD across rodent strain, suggesting substantial heritability of DRD.

To date, four human studies have assessed the heritability of delay discounting and all four identified evidence of heritability.

Early adolescent twins were found to have genetic influences on DRD at ages 12 (30%) and 14 (51%, Anokhin et al., 2011). Additionally, in a sample of 17-year-old twins, strong evidence of heritability was found in two different DRD phenotypes (47–51%, Isen et al., 2014; Sparks et al., 2014). Most recently, Anokhin et al. (2015) assessed DRD in a sample of twins and found significant heritability of both DRD indices (AUC: 46 and 62%; *k*: 35 and 55% at age 16 and 18 respectively). The trend of increasing genetic influence in later adolescence is likely attributable to ongoing adolescent brain maturation of prefrontal regions implicated in intertemporal choice (Carter et al., 2010; Steinberg, 2010; Peters and Büchel, 2011; Luo et al., 2012). Taken together, both animal and human studies suggest that DRD is heritable and possesses similar rates of heritability as addiction phenotypes (i.e., ∼50%).

In the domain of family history, rodent studies support the presence of elevated levels of DRD in non-affected family members (as compared to the general population). Specifically, three studies to date of alcohol-naïve rodents selectively bred for high- or low-alcohol preference, found that high-alcohol preferring subjects exhibited an increased rate of DRD of sucrose rewards (Wilhelm and Mitchell, 2008; Oberlin and Grahame, 2009; Perkel et al., 2015). Notably, one study did not find a difference in DRD of sucrose rewards between high- and low-alcohol preferring rodents (Wilhelm et al., 2007). Nonetheless, the majority of evidence suggests that heritability for alcohol abuse susceptibility overlaps with heritability for DRD preference, and that in subjects susceptible to alcohol abuse, impulsive DRD is present prior to alcohol exposure.

While human research has been mixed regarding the presence of DRD at elevated rates in non-affected family members, earlier studies suffered from significant methodological issues (most notably, small sample size; e.g., Crean et al., 2002; Petry et al., 2002; Herting et al., 2010). A more recent highly-powered study found that in 298 healthy young adults (age $M = 23$), those with a family history positive for alcohol or other drug use disorders had higher rates of DRD (Acheson et al., 2011). Furthermore, the study found that impulsive DRD was significantly associated with having more parents and grandparents with alcohol and drug use disorders. Similarly, Dougherty et al. (2014) found that in 386 non-affected youth (ages 10–12), those with family histories of alcohol or other drug use disorders had higher rates of DRD. These findings suggest that in studies with adequate power and a thorough assessment of family history of substance use disorders, there is evidence that non-affected family members of individuals with substance use disorders possess higher rates of DRD than the general population. Similarly, this body of research suggests that given the overlap in heritability of drug abuse and impulsive DRD, there is likely an overlap of specific genetic loci conferring risk for drug abuse and for DRD.

Relatively recent efforts have been made to determine the molecular genetic basis of DRD, primarily within dopaminergic genes. Currently, findings primarily suggest the involvement of the single nucleotide polymorphisms (SNPs) from *COMT* (rs4680) and *ANKK1* (rs1800497), and the exon 3 variable number of tandem repeats (VNTR) polymorphism in *DRD4*, genes which are all implicated in dopamine neurotransmission (Boettiger et al., 2007; Eisenberg et al., 2007; Paloyelis et al., 2010; Gianotti et al., 2012; Smith and Boettiger, 2012; Gray and MacKillop, 2014). Regarding rs4680, four studies found an association between possession of the G allele and impulsive DRD in adults (Boettiger et al., 2007; Gianotti et al., 2012; Smith and Boettiger, 2012; MacKillop et al., in press), one found an association of A/A with impulsive DRD in young adults (Paloyelis et al., 2010), and another found no association (Gray and MacKillop, 2014). The A/A genotype of rs4680 is associated with a reduction in levels of catechol-*O*-methyl transferase enzymatic activity (an enzyme implicated in dopamine catabolism), which leads to higher levels of dopamine primarily in the prefrontal cortex (Weinshilboum et al., 1999; Chen et al., 2004; Tunbridge et al., 2004). Gianotti et al. (2012) found that reduced activity in the left dorsal prefrontal cortex (dPFC) during a resting state paradigm mediates the effect of the G allele on impulsive DRD (also see Boettiger et al., 2007). This suggests that the G allele of rs4680 reduces baseline dPFC engagement via reduced dopamine availability, leading to more impulsive decision making. The dPFC does indeed appear to be strongly implicated in impulsive decision making as it is known to impact self-control processes (Gianotti et al., 2009; Knoch et al., 2010) and the dorsolateral prefrontal cortex (dlPFC) has been shown to affect DRD rates when stimulated transcranially (discussed below). Future studies with large healthy populations are required to verify which genotype is of greatest risk and examine moderators (e.g., age effects), as one recent study's findings suggest a U-shape curve between dopamine levels and DRD performance (i.e., too much or too little dopamine yields impulsive DRD; Smith and Boettiger, 2012). Nonetheless, current research supports a relationship between *COMT* (rs4680) and DRD rates via dPFC dopamine levels.

The T allele of rs1800497 has been associated with DRD in two studies (Eisenberg et al., 2007; MacKillop et al., in press), and not associated in two others (Kawamura et al., 2013; Gray and MacKillop, 2014). However, considerable heterogeneity in sample demographics (e.g., healthy college students, weekly gamblers, healthy adults) and sample sizes (between 91 and 195 participants) may explain the mixed findings. The role of the rs1800497 SNP is less well understood because it is technically in the *ANKK1* gene, near the *DRD2* gene. However, rs1800497 is in high linkage disequilibrium with SNPs from multiple genes in this region (*NCAM1-TTC12-ANKK1-DRD2*, Mota et al., 2012) and is associated with dopamine D_2 receptor density (Pohjalainen et al., 1998; Jönsson et al., 1999; Savitz et al., 2013). Regardless of the specific mechanism of influence of rs1800497, its association with dopamine availability and with multiple addictive genotype influences (for a review see Ma et al., 2014) suggests it should be investigated further in relation to DRD rates.

DRD4 VNTR influences intracellular levels of cyclic adenosine monophosphate to primarily impact dopamine response in the prefrontal cortex, however, the specific downstream biochemical impact of different variants of *DRD4* VNTR remains relatively unclear (Oak et al., 2000) and recent studies have examined

the role of rare variants rather than length of repeats (e.g., Tovo-Rodrigues et al., 2012; Michealraj et al., 2014). *DRD4* VNTR and DRD has been explored in several studies, with mixed findings, and appears to have a more context dependent relationship with DRD rates. For example, one study found the combination of the long form of *DRD4* VNTR and the T allele of rs1800497 to be associated with significantly higher DRD rates (Eisenberg et al., 2007), and a second study found increased DRD rates in low socioeconomic status (SES) long form carriers versus decreased DRD rates in mid-to-high SES long form carriers (Sweitzer et al., 2013). In addition, studies have reported a direct negative relationship between the long from and decreased DRD rates (Gray and MacKillop, 2014) and no direct association (Eisenberg et al., 2007; Garcia et al., 2010; Paloyelis et al., 2010; Sweitzer et al., 2013). However, the existing studies have varied widely in sample composition (e.g., healthy college students, adolescents with attention deficit hyperactivity disorder [ADHD]) and size (ranging from 68 to 546). It will be important for future studies to continue to explore the potential of *DRD4* VNTR as a differential susceptibility gene (see Bakermans-Kranenburg and van Ijzendoorn, 2011) in order to determine whether the relationship between DRD and polymorphisms of varying length or rarity is contingent upon other genes or environmental stressors.

Despite some promising findings regarding the role of *COMT*, *DRD2*, and *DRD4*, the associations require consistent replication and the effect sizes have been relatively small. Nonetheless, current empirical findings and theory suggest a central involvement of dopamine functioning as well as possible interactions among serotonin and dopamine systems on DRD performance (Winstanley et al., 2005; Simon et al., 2013). Greater exploration of other systems related to reward processing as well as genome-wide association studies are a priority for future research. Identification of robust genetic correlates of DRD would provide insights into the neurobiological causes of variation, identifying targets for possible pharmacological and neuromodulatory interventions.

Taken together, DRD is relatively well supported as an endophenotype for addictive disorders, although the identification of specific polymorphisms responsible for variation is nascent. The initial molecular genetic studies suggest that dopamine transmission plays an important role in DRD, yet in almost all cases, the candidate loci were the 'usual suspects' (i.e., loci tested most frequently for associations with addictive behavior and other externalizing psychopathology). Future work that establishes the robustness of these findings and expands the genomic perspective will be essential.

Interventions Targeting Delayed Reward Discounting

Several experimental manipulations have been examined for reducing high rates of DRD, and can be broadly classified into three domains: behavioral interventions, pharmacological interventions, and neuromodulatory manipulations using transcranial stimulation of specific brain regions.

Behavioral Interventions

The earliest research exploring the link between distraction and DRD was conducted on preschool age children (3–5 years old) who underwent the aforementioned marshmallow test. In this early work, when encouraged to think of other things or play with toys, the children more frequently waited longer for the delayed reward (Mischel and Ebbesen, 1970; Mischel et al., 1972). Similar findings have been identified in animal studies (Grosch and Neuringer, 1981; Evans and Beran, 2007). This is thought to operate similarly to distraction manipulations that lead to more effective resistance to food or drug cravings in susceptible individuals (e.g., Versland and Rosenberg, 2007; Van Dillen et al., 2013; Murphy and MacKillop, 2014). However, it remains unclear whether distraction techniques can offer long-term (rather than merely temporary) disruption of immediate reward pursuit (see Ashe et al., 2015).

The converse of distraction-based techniques is a mindfulness approach which seeks to encourage non-judgmental and objective monitoring of one's own thoughts and behaviors in an effort make well considered, unimpulsive decisions (Marlatt, 2002). For example, one study employed a brief 60–90 min training based on Acceptance and Commitment Therapy (Hayes et al., 2011; Morrison et al., 2014). In this training session, subjects discussed internal barriers to healthy decision making with a therapist and engaged in several exercises designed to aid the participant in observing their emotions and learning to act on values rather than feelings. Participants who were engaged in this training procedure exhibited decreases in DRD, whereas waitlist controls did not. Brief mindfulness-based interventions have been adapted for obese individuals and have shown efficacy for reducing DRD of food items (Hendrickson and Rasmussen, 2013). In a similar spirit of priming mindfulness, Berry et al. (2014) found that preemptive and concurrent visual exposure to natural environments (e.g., mountains), led to approximately a 50% reduction in DRD compared to decisions made during exposure to built environments (e.g., buildings) and control environments (e.g., triangles). All of the work in this area has focused on acute outcomes and it will be important for future studies to explore longitudinally how mindfulness training may influence long-term decision making patterns.

Beyond distraction and mindfulness, a wide variety of other behavioral techniques have been applied to reduce impulsive discounting. For example, a small number of early studies employed a fading procedure with pigeons that gradually increased the delay between the small reinforcer and the larger delayed reinforcer, which yielded an increased selection of the delayed reward (Mazur and Logue, 1978; Logue et al., 1984). Similar studies have been conducted primarily in children with conditions associated with impulsivity (e.g., mental retardation, autism, ADHD) and have found reductions in DRD (Schweitzer and Sulzer-Azaroff, 1988; Dixon et al., 1998; Fisher et al., 2000). In the context of these experiments, the participants were offered a small immediate reinforcer or a large reinforcer that was contingent on engagement in a target behavior (e.g., staying seated) for a required duration. The duration for performing the target behavior was gradually increased overtime and the children typically showed increasing ability to maintain this behavior for

extended periods of time in order to obtain the larger reinforcer. However, sample sizes ranged from 3 to 6 participants and the applicability of this technique to healthy adolescents (a typical target sample for drug abuse prevention) or substance using adults is relatively low.

Another method of reorienting individuals toward larger delayed rewards is "reward bundling," or grouping a series of DRD choices into a single decision. For example, for the "reward bundling" condition, one recent study informed participants that if they choose a smaller sooner reward then they will receive that reward every 2 weeks after that for 6–10 weeks, and if they choose a larger delayed reward in 10 days then they will receive that reward every 2 weeks after that for 6–10 weeks (Hofmeyr et al., 2011). This makes theoretical sense, as orienting individuals to considering a series of consequences of a pattern of decision making (as opposed to a consequence derived from a single choice) may increase their consideration of avoiding a sum of reduced rewards by choosing to favor larger greater rewards. For example, if the choice to get intoxicated now at the cost of feeling good tomorrow were to entail commitment to this same choice every day for the next week, the value of the larger delayed reward relative to the smaller sooner reward would presumably increase (Monterosso and Ainslie, 2007). Bundling has been supported empirically by several laboratory studies involving animals and humans (Kirby and Guastello, 2001; Ainslie and Monterosso, 2003; Hofmeyr et al., 2011; Stein et al., 2013). The most recent human study, conducted by Hofmeyr et al. (2011), found that smokers, but not non-smokers, were particularly susceptible to the reward bundling manipulation. This suggests that those who are more susceptible to addictive behavior may be in greater need of and more responsive to interventions that challenge them to consider the long-term aggregation of rewards. Relatedly, a study found that in cocaine and/or alcohol outpatient substance users, an intervention comprised of individual counselor-facilitated training in monthly budgeting, which focused on long term goals and limited short-term spending, led to a decrease in both DRD and cocaine use (Black and Rosen, 2010).

Another strategy for modifying discounting is episodic future thinking, which is a method of increasing future orientation by prompting individuals with autobiographical, emotional, and circumstantial details that are expected to occur at specified delays in the future (Atance and O'Neill, 2001). For example in two fMRI experiments, Peters and Büchel (2010) found that when delays were paired with events the subjects were likely to engage in during that time (e.g., "20€ now or 35€ in 45 days (vacation Paris)"), subjects were more likely to choose the delayed rewards than when rewards were not presented with these tags. This finding has been replicated in three additional studies (Benoit et al., 2011; Daniel et al., 2013a,b). Most recently, a study found that episodic future thinking is not dependent on positive affect induction for its effects rather, even neutral-valenced events shift time perspective to reduce DRD (Lin and Epstein, 2014). Using a conceptually similar strategy, one investigation conducted four studies utilizing virtual reality to display computerized renderings of participants' future selves, and in all cases they found that those who interacted with their virtual future selves had reduced DRD (Hershfield et al., 2011). This represents a promising method for engaging individuals in greater imagination of their future in order to reduce DRD.

From a more purely cognitive standpoint, a phenomenon that has been demonstrated in several studies is framing effects, or the tendency of DRD to fluctuate in relation to the specific wording of the delay. Read et al. (2005) first demonstrated that when delays are framed as calendar dates (e.g., on December 5), discount rates tend to decrease and the shape of the discount function becomes more linear (less hyperbolic). Other studies have had similar findings (LeBoeuf, 2006; Klapproth, 2012; DeHart and Odum, 2015). Notably, other variables involved in question framing have been shown to either decrease DRD, such as presenting participants with an explicit zero paired with the options (e.g., "[A] $5.00 today and $0 in 26 days OR [B] $0 today and $6.20 in 26 days"; Magen et al., 2008). A common element across all of these formats is that they seem to increase the salience of the delay by framing the specific date (possibly increasing the perceived likelihood of actually receiving the reward; see Patak and Reynolds, 2007), increasing attention to the notion that they will receive "$0" at the delay if they select the immediate reward.

Finally, strengthening the elementary cognitive processes that subserve DRD decision making represents a further strategy for reducing this form of impulsivity. Two studies have been conducted to explore the extent to which working memory training can improve overall executive functioning capabilities as a way to decrease DRD and improve overall decision making capabilities. The first study randomized a small number of individuals ($N = 27$) into a training condition and a matched control condition (Bickel et al., 2011). The working memory training used a computer program consisting of several challenges (e.g., recalling a sequence of digits forward or backward) administered 4–15 times over the course of approximately 25 days. In the control condition, participants were exposed to the same set of stimuli, but were provided with the answers so that they did not need to engage their working memory. The study found that the working memory training group significantly decreased discounting rates by approximately 50%, whereas the control group exhibited no significant reductions in DRD. A recent study did not replicate the connection between working memory training and reduced DRD in a rodent model (Renda et al., 2015), although major methodological differences were present (e.g., species, type of task, prior substance use). Ameliorating delay discounting via working memory is at an early stage but has considerable promise.

Pharmacological Interventions

Several studies have tested the efficacy of dopamine (DA) agonists (e.g., amphetamine) and DA-norepinephrine (NE) agonists (e.g., methylphenidate) for reducing DRD. Frequently prescribed to individuals with ADHD, amphetamine and methylphenidate are thought to increase executive functioning capacity by facilitating transmission of catecholamines in critical regions (Bidwell et al., 2011). Studies have found that both amphetamine and methylphenidate typically reduce DRD in rat models

(Cardinal et al., 2000; Adriani et al., 2004; van Gaalen et al., 2006; Bizot et al., 2011), however, null or even opposite effects have occasionally been detected when varying methodology (e.g., rearing environments, signaled or unsignaled rewards; Cardinal et al., 2000; Perry et al., 2008). Of the human studies that have been conducted, one found that amphetamine decreased DRD in healthy adults (de Wit et al., 2002), and the others found that methylphenidate decreased DRD in a sample of adults with a criminal background (Pietras et al., 2003) and in a sample of children with ADHD (Shiels et al., 2009). Despite the promise of these human studies, the therapeutic use of these substances in reducing DRD must be balanced with their high abuse potential in individuals without ADHD (Kollins, 2007).

Additional compounds have been examined for efficacy in reducing DRD, including compounds with less direct and concentrated effects on DA availability, such as NE agonists (e.g., atomoxetine), adrenergic agonists (e.g., guanfacine), and multiple monoamine agonists (e.g., modafinil; Wilens, 2006). These substances are of particular interest because they uniformly exhibit minimal abuse potential (Malcolm et al., 2002; Muir and Perry, 2010; Upadhyaya et al., 2013). Among these three compounds, atomoxetine has been studied most extensively, but only in rodent models to date. Early research by Robinson et al. (2008) found that atomoxetine significantly decreased several forms of impulsivity, including DRD. Similarly, Bizot et al. (2011) found that subjects given atomoxetine were more likely to select the large but delayed reward. However, other studies have found atomoxetine increased DRD in healthy rodents (Broos et al., 2012), or had no effect on DRD in healthy (Baarendse and Vanderschuren, 2012), spontaneously hypertensive (an animal model for ADHD; Turner et al., 2013), and cocaine-withdrawing rodents (Broos et al., 2014). In the latter study, despite no changes in DRD, the rodents were less likely to readminister cocaine at 1 and 10 days (Broos et al., 2014). Finally, one study found that chronic atomoxetine treatment during adolescence (but not acute atomoxetine in adulthood) led to a stable decrease in DRD when tested in adulthood, suggesting lasting effects of the atomoxetine in the orbitofrontal cortex (Sun et al., 2012).

Early studies testing effects of guanfacine and modafinil on DRD are promising. One study found that intramuscular guanfacine reduced DRD in rhesus monkeys (Kim et al., 2012) and a second found dose-dependent reduction in DRD in rats when guanfacine was administered locally in the ventral hippocampus (Abela and Chudasama, 2014). In a fMRI study on humans, modafinil was found to decrease DRD in alcohol dependent participants, but yielded no change in healthy control subjects, suggesting that modafinil normalizes DRD decision making in alcohol dependent patients (Schmaal et al., 2014). Moreover, reductions in DRD were accompanied by an enhanced functional connectivity between the superior frontal gyrus and ventral striatum, suggesting more prefrontal control over these choices. It will be important for future studies to continue to examine the effects of these medications on DRD, particularly in humans with and without high levels of DRD, to establish the consistent and stable effects.

Neuromodulatory Manipulations

Recent efforts have been made to explore the impact of human non-invasive transcranial brain stimulation on DRD, both through magnetic and direct electrical current stimulation. Transcranial magnetic stimulation (TMS) operates by passing electricity through a coil placed near the region of focus. The resulting magnetic field can be used to temporarily modulate brain activity in nearby regions. One group assessed the effect of dlPFC interruption during a DRD task as well as a single item valuation task (i.e., participants rated the attractiveness of 12 single-options taken from the DRD choice set; Figner et al., 2010). They found that left (but not right) dlPFC inhibition increased impulsive responding on the DRD task, but neither region impacted item valuation on the single item valuation task. This suggests that the left dlPFC is critical to self-control (inhibiting responses for salient immediate rewards) rather than in stimulus value appraisal. However, inhibition of the right dlPFC was observed to reduce impulsive DRD in another study (Cho et al., 2010), but only when participants were exposed to a significantly higher frequency and shorter duration of magnetic stimulation than in the study by Figner et al. (2010). Additionally, a subsequent study using positron emission tomography (PET) found that inhibition of the right dlPFC reduced impulsive DRD rates (reducing impulsivity) and disrupted regional cerebral blood flow (rCBF) in the right dlPFC and right rostral PFC leading to diminished correlations between DRD rates and rCBF of these (and other) prefrontal regions (Cho et al., 2012). This suggests that the neural network underlying impulsive decision making is disrupted by right dlPFC inhibition. Finally, also using PET imaging, stimulation of the medial prefrontal cortex has been found to both reduce DRD rates and reduce the level of synaptic dopamine in the striatum (Cho et al., 2015). This is clearly a mixed literature and discrepancies among these findings will need to be reconciled in future studies.

In contrast to TMS, transcranial direct current stimulation (tDCS) operates by directly passing electrical currents to surface electrodes placed on the scalp proximal to the region(s) of interest. One study found no effect of inhibition of left dlPFC and stimulation of right dlPFC on DRD, but did find that when the right dlPFC is inhibited and the left dlPFC is stimulated, impulsive DRD rates increase (Hecht et al., 2013). A second study also found no effects of left dlPFC inhibition and right dlPFC stimulation (Kekic et al., 2014). Clearly, this work is at an early stage and future research should seek to replicate and clarify these findings. Additional priorities include further exploration of the role of anode/cathode placement, electrode size, and current intensity on DRD rates.

Despite early studies showing promise of non-invasive brain stimulation of the prefrontal cortex in improving impulsive decision making, it will be important for future studies to clarify the ideal tool for stimulation (i.e., TMS or tDCS), frequency and intensity of stimulation, and manipulation of left and right prefrontal areas. Although research is nascent, TMS appears to have support both from aforementioned studies and from interventions for related cognitive functions. For example, a recent meta-analysis assessed the efficacy of TMS and tDCS of the dLPFC to improve working memory performance (Brunoni and

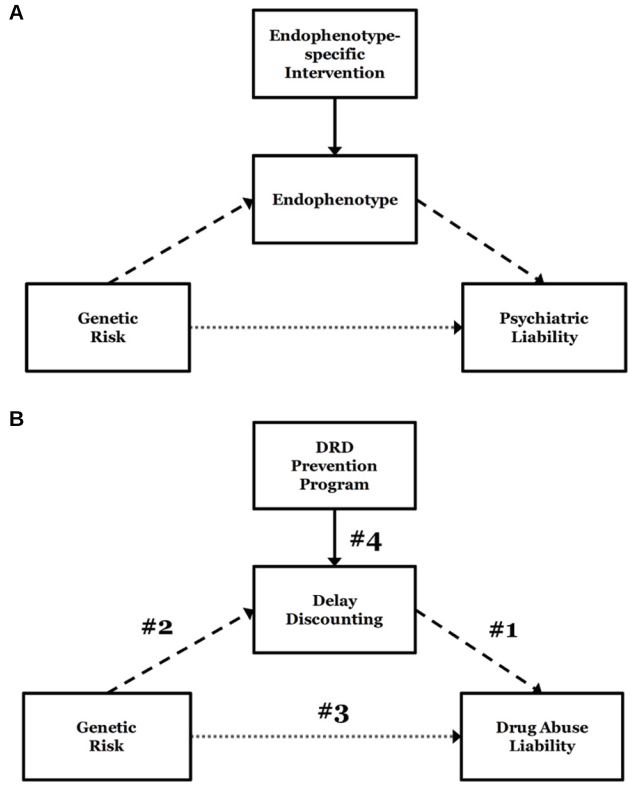

FIGURE 2 | A framework for considering developing genetically-informed prevention and treatment. General theoretical framework for an endophenotype approach to addiction genetics, integrating endophenotype-specific treatment approaches (Ray et al., 2010) **(A)**. Specific model of delayed reward discounting (DRD) as an endophenotype of genetic risk for addiction liability **(B)**. Numbers 1–4 reflect the necessary components to establish DRD as a viable genetically-influenced prevention target for drug abuse: (1) a robust relationship between DRD and drug abuse; (2) genetic loci that are reliably associated with delay discounting; (3) possession of risk alleles for DRD risk that are responsible for drug abuse risk; (4) efficacious strategies for reducing impulsive DRD.

Vanderhasselt, 2014), a cognitive function that has been linked to DRD performance (Bickel et al., 2011; Wesley and Bickel, 2014). They found that TMS improved response time and accuracy whereas tDCS improved only response times. Several possible mechanisms may explain differences in effects found such as difference in study design, equivalency of "doses," and better spatial precision of TMS. In addition to identifying optimal stimulation methodology, it will be important for future studies to explore the potential long term effects of transcranial stimulation treatment for DRD.

Critique of Delayed Reward Discounting as a Genetically-Informed Drug Abuse Prevention Target

In the preceding sections, we reviewed impulsive DRD as a behavioral characteristic, evidence linking it to genetic influences, and evidence that it can be significantly ameliorated using a number of strategies. Here, we consider the assembled findings and the specific question of whether it has promise for genetically-informed drug abuse prevention. To do so, we provide a framework for considering the links that are necessary for coming to that conclusion (**Figure 2**). The framework is an extension of a previous model for integrating alcohol endophenotypes into treatment development and prospective pharmacotherapies (Ray et al., 2010). As depicted in **Figure 2A**, we propose that endophenotypes can enhance the prospect of genetically-informed intervention of any kind (prevention or treatment) by identifying genetically-mediated risk mechanisms that both enhance the resolution of risk status and serve as intervention targets. In the first case, endophenotypes (e.g., impulsive DRD) are anticipated to ultimately lead to more reliable identification of risk alleles via more robust relationships with individual genotypes. In the second case, the identification of risk status in the context of a specific mechanism implicitly reveals a candidate intervention target. In other words, endophenotypes have the potential to elucidate both the biological causes of the disorder and provide personalized intervention targets.

Figure 2B lays out the case for DRD, identifying the four links that necessarily comprise an argument for genetically-influenced prevention programming. These can be summarized by the following questions:

(1) *Is DRD reliably associated with drug abuse?*
(2) *Are specific genetic loci reliably associated with DRD?*
(3) *Are DRD risk alleles also responsible for drug abuse risk?*
(4) *Are there established strategies for reducing impulsive DRD?*

If the empirical support for the links in **Figure 2B** is reliably present, the case for a genetically-informed DRD prevention strategy would be entirely sound. Where those links are less than robust, however, there remain ambiguities and open questions, and the rationale becomes more debatable.

In light of the preceding sections, it is clear the latter is the case for DRD. In the discounting framework, the strongest link is the first, the association between impulsive DRD and drug abuse. As discussed, this relationship has been observed in an array of different samples with an array of different methods, cross-sectionally and longitudinally. With regard to the genetic linkages (2 and 3), the literature remains at an early stage. As noted earlier, although there is relatively strong evidence that DRD is heritable, there is not a sufficiently strong basis for defining individuals at higher or lower genetic risk based on individual genotypes or multi-locus risk scores at this time. Furthermore, there is very limited evidence that discounting risk alleles indirectly impact addictive behavior; this has only been directly demonstrated in one study (Gray and MacKillop, 2014). Similarly, at the level of link 4, intervention research on DRD remains incipient. Although several methods have been applied to reducing DRD rates, there is limited consensus on the ideal approach or combinations of approaches. Furthermore, many of the methods have not been replicated, examined for prolonged reductions, or tested in adolescent populations that would be most appropriate for drug abuse prevention. Similarly, it is clear is that a number of different strategies are effective under controlled experimental conditions, but not clear which strategies (or package of strategies) will successfully translate

from the laboratory to 'live' interventions producing long-term changes. In sum, a full implementation of an evidence-based DRD prevention program for individuals who are genetically at-risk for more impulsive discounting (and thereby addictive disorders) is simply not supported by current literature.

Where these links are weakest are the future priorities for the field. Progress in more definitively identifying genetic correlates of DRD is essential. Equally, a leading priority going forward is for pilot research to determine the utility of the discounting reduction strategies in adolescent samples to identify promising strategies for prevention contexts. Such studies would be well-suited to focus on proximal outcomes, most obviously DRD itself, drug-related motivation, and short-term substance abuse outcomes. The basis for presuming downstream positive effects of reducing DRD, of reorienting an individual away from immediate impulses and toward making more future oriented decisions, is a logical step forward. Developing efficacious interventions holds wide implications, not only for addictive disorders, but also more broadly on behaviors such as good nutrition, financial planning, and health maintenance behaviors that impact wide swaths of the general public (Howlett et al., 2008; Bradford, 2010; Epstein et al., 2010).

An important step in the future will be to identify standardized norms for DRD performance. As discounting is typically only assessed in research contexts, there is currently no basis for determining who should be targeted as a result of their DRD performance. In order to provide secondary prevention programming, it is necessary who is and who is not at-risk. The absence of normative data is a prosaic but nonetheless significant impediment to progress in this area.

The final point worthy of discussion is whether targeting discounting based on genetic profiles is a worthwhile undertaking more broadly. Certainly, from the perspective of personalized medicine, optimization of any approach using idiographic data (genetic or otherwise) is desirable. However, given the small and inconsistent relationships between risk alleles and impulsive DRD, as well as the extra step involved in genotyping individuals, a more feasible alternative would be using standardized normative data for risk identification rather than genetic risk profiles. In addition, it is also notable that psychosocial factors, such as early life stress, have also been associated with more impulsive discounting (e.g., Lovallo et al., 2013) and may be useful for risk identification. In other words, at the current time, there is a much stronger rationale for an efficacious DRD prevention program to be deployed for individuals who are in high-risk groups or exhibit DRD rates that significantly deviate from standardized norms than based on genotype. Alternatively, among young adults, the misuse of alcohol, tobacco, and other drugs is so prevalent, indeed almost normative in the case of alcohol, and the links between DRD and diverse forms of externalizing behavior are so robust, that primary prevention (i.e., universal) may be a more appropriate strategy. Intervention matching and secondary prevention are typically assumed to be desirable to maximize impact and efficiency, but, in this case, if an efficacious multi-component impulsive DRD prevention program can be developed, it will be of relevance to the large majority of young adults.

Conclusion

The goal of this review was to evaluate the viability of DRD as a target for addictive disorders from the perspective of genetically-informed drug abuse prevention. A large body of research links impulsive DRD to drug abuse and supports the hypothesis that DRD is an endophenotype for addictive disorders. Additionally, current findings suggest that there are multiple promising methods—behavioral, pharmacological, and neuromodulatory—for acutely reducing DRD. However, the evidence for long-term changes and subsequent salutary health benefits is scant and no studies have directly assessed preventive interventions for impulsive DRD. Although significant gaps in knowledge remain and the wisdom of the long-term goal of genetically-informed drug abuse prevention via DRD is debatable, the current state of the science nonetheless suggests a more cautious conclusion, that impulsive DRD is more generally a promising target for drug abuse prevention and specific empirical investigations in this area are warranted.

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The relationship between alcohol consumption, perceived stress, and CRHR1 genotype on the hypothalamic–pituitary–adrenal axis in rural African Americans

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Objective: Rurally situated African Americans suffer from stress and drug-related health disparities. Unfortunately, research on potential mechanisms that underlie this public health problem have received limited focus in the scientific literature. This study investigated the effects of perceived stress, alcohol consumption, and genotype on the hypothalamic–pituitary–adrenal (HPA) Axis. **Methods:** A rural sample of African American emerging adults ($n = 84$) completed a battery of assessments and provided six samples of salivary cortisol at wakeup, 30 min post wakeup, 90 min post wakeup, 3:00 PM, 3:30 PM, and 4:30 PM. **Results:** Participants with a TT genotype of the CRHR1 (rs4792887) gene tended to produce the most basal cortisol throughout the day while participants with a CC genotype produced the least amount. Increased levels of perceived stress or alcohol consumption were associated with a blunted cortisol awakening response (CAR). Moreover, the CAR was obliterated for participants who reported both higher stress and alcohol consumption. **Conclusion:** Perceived stress and alcohol consumption had a deleterious effect on the HPA-Axis. Furthermore, genotype predicted level of cortisol production throughout the day. These findings support the need to further investigate the relationship between stress dysregulation, drug-use vulnerability, and associated health disparities that affect this community.

Keywords: African Americans, alcohol, HPA-axis, stress, CRHR1, health disparities

Introduction

African Americans are disproportionately exposed to chronic stress by way of high levels of racism, discrimination, violence, crime, neighborhood disorganization, unemployment, financial strain, and low socioeconomic status (Clark et al., 1999; Dalaker, 2001; Centers for Disease Control and Prevention, 2004, 2005; Brody et al., 2015; Myers et al., 2015). Persistent exposure to chronic stress causes "wear-and-tear" on the body's regulatory system and compromises its capacity to recover efficiently from incessant exposure to environmental stressors (McEwen, 1998). Consequent

alterations in stress responsive systems is theorized to heighten drug-use vulnerability as drug use provides a coping strategy for alleviating negative affect that is becoming increasingly more difficult to manage and temporally normalizes stress physiology (Koob and Le Moal, 2001, 2008). Subsequently, drug abuse and dependency—compounded by a dysregulated stress system—places African Americans at-risk for disproportionate levels of drug use consequences associated with violence (e.g., homicide, suicide, child abuse, and domestic violence), injuries (e.g., crashes, falls, burns, and drowning), and health disparities (e.g., prostate cancer, liver cancer, chronic liver disease, hypertension, myocardial infarction, gastritis, pancreatitis, STDs, meningitis, and poor control of diabetes; Naimi et al., 2003; National Institute on Drug Abuse, 2003; Centers for Disease Control and Prevention, 2004). The purpose of this study was to investigate the effects of stress, alcohol consumption, and genotype on the hypothalamic–pituitary–adrenal (HPA) axis.

The body responds to internal and external stressors via the stress-response system or HPA-axis. Drug use has been linked to the stress-response system in both rats (Coste et al., 2000) and humans (Lovallo et al., 2000; Fox et al., 2007). Specifically, dysregulation of the HPA has been associated with drug dependence, withdrawal, and relapse (Koob and Le Moal, 2001; Lovallo, 2006). However, the underlying mechanisms for this link remain unclear (Sommer et al., 2008). Recent investigations have considered the role of the corticotropin-releasing hormone (CRH) system as a critical modulator of HPA stress reactivity. Of particular focus, is the CRH receptor 1 (CRHR1) genotype. Specifically, CRHR1 is a 7-transmembrane G-protein-coupled receptor that is expressed in high density in the cerebral cortex, cerebellum, hippocampus, amygdala, and pituitary; and peripherally in the skin, ovaries, testes, and adrenal gland (Binder and Nemeroff, 2010).

Corticotropin releasing hormone receptor 1 is thought to be the principal receptor mediating the stress response and is highly expressed in the anterior pituitary, neocortex, hippocampus, amygdala, and cerebellum (Timpl et al., 1998). Furthermore, it has been found to be associated with the pathophysiology of anxiety, including PTSD, depression, and heavy alcohol consumption (Treutlein et al., 2006; Bradley et al., 2008; Wasserman et al., 2008, 2009; Binder and Nemeroff, 2010; Amstadter et al., 2011; Enoch, 2011). It has also been found to modulate dopamine function, ethanol-induced enhancement of GABAergic synaptic transmission, and reward learning (Nie et al., 2004; Bogdan et al., 2011). CRHR1 knockout rat models have demonstrated that lacking CRHR1 significantly reduces the release of adrenocorticotropic hormone (ACTH) and corticosterone; resulting in an impaired stress response that cannot be compensated by any other system (Timpl et al., 1998). Extending these findings to human studies may illustrate how CRHR1 genotype could predict genetic vulnerabilities that have a direct bearing on the production of hormonal biomarkers in response to environmental demands.

Cortisol, a glucocorticoid, is a hormonal biomarker of HPA functioning (Frodl and O'Keane, 2013). Cortisol is produced in the adrenal cortex and has a relatively stable diurnal rhythm that is characterized by a sharp increase within 30 min of morning waking, followed by a steep decline by mid-morning, and gradual decline during the course of the day (Stone et al., 2001; Shirtcliff et al., 2012). This pattern is an important component of HPA regulation, as high morning levels help individuals prepare for the day's events, and low evening levels permit critical immune and tissue repair (Dallman et al., 2002). Investigating this diurnal pattern provides a window into the extent to which the body's stress response system is altered as a function of chronic stress (McEwen, 2004). Consistent with McEwen's (2004) concept of allostasis, or longstanding changes in the set-points of the stress system, the individual is theorized as being capable of adapting or changing to meet the demands of a changing environment or social context. As the individual is consistently exposed to that environment over time, the inherent variability may become less malleable. Allostasis frames the interplay between genetic, neural, behavioral and environmental forces as a developmental phenomenon with social contextual cues shaping the phenotypic expression of the genetic and biological building blocks. A specific marker of the functioning of the HPA-axis is the cortisol awakening response (CAR).

It is estimated that the CAR represents a 40–75% increase in cortisol 20–45 min post waking (Wust et al., 2000; Chida and Steptoe, 2009; Fries et al., 2009; Zeiders et al., 2014). After the CAR, the morning portion of the diurnal rhythm is principally controlled by the anterior pituitary and is under strong genetic influence (Van Hulle et al., 2012). Individual differences in the CAR is thought to be a reliable indicator of HPA functioning specifically, and a correlate of psychological and physical health broadly (Chida and Steptoe, 2009; Gonzalez et al., 2009; Thorn et al., 2009; Zeiders et al., 2014). More specifically, a hyperactive CAR has been linked to experiences of general life stress, overload, and worrying (Wust et al., 2000; Chida and Steptoe, 2009; Fries et al., 2009). Furthermore, a hypoactive CAR has been linked to chronic stress, PTSD, fatigue, burnout, depression, and hopelessness (Chida and Steptoe, 2009; Fries et al., 2009). A blunted CAR has also been linked to poor health outcomes like cardiovascular, autoimmune, atopic, and psychiatric disease (Fries et al., 2009). Taken together, increases in CAR suggest a sense of arousal that is garnered to meet upcoming challenges while decreases in CAR may reflect a more dysregulated HPA as a function of chronic exposure to debilitating stress (Thorn et al., 2009).

Chronic activation of stress responsive systems by ongoing experiences of racism, violence, crime, unemployment, financial strain, and low-to-no socioeconomic status can cause "wear and tear" on regulatory systems by way of allostatic load (McEwen, 1998). Allostasis does not provide a simple prediction for unidirectional alterations, with both hypo- and hyper-arousal resulting from extreme environmental input (Gunnar and Vazquez, 2001). Salient stressful experiences may alter the "setpoint" for stress regulation along hypo- or hyper-arousal trajectories. A dysregulated stress system, in turn, may contribute to drug use and abuse (Koob and Le Moal, 2001).

The relationship between the dysregulation of the HPA-axis and drug addiction has led to mixed research findings. Previous research suggests that alcohol, cigarettes, and cocaine increase HPA activity (Adinoff et al., 1998, 2003; Goeders,

2002; Mendelson et al., 2005). Conversely, heroin addicts have been found to exhibit a hyporesponsive HPA, lower cortisol levels in basal condition, and reduced cortisol decrease in the evenings (Facchinetti et al., 1985; Kreek et al., 2005). Animal models suggest that sensitivity to stress, recovery from stress, and/or the uncontrollability of stressors, are strong predictors of drug use and abuse (Goeders and Guerin, 1994; Campbell and Carroll, 2001; Goeders, 2002). As the adrenal cortex begins to produce more cortisol, the individual may be more sensitized to use alcohol, potentially establishing a positive-feedback loop where cortisol and psychological stress increase motivation to use alcohol (Bernardy et al., 1996; Adinoff et al., 1998; Thayer et al., 2006).

To date, animal models have driven the direction of experimental research investigating the link between stress dysregulation and drug abuse. Rasmussen et al. (2001) found that chronic alcohol consumption produced neuroendocrine and behavioral responses in rats that may increase risk for future abuse. The activation of the HPA-axis and enhanced expression of CRH have been observed during acute phases of drug dependence withdrawal, including benzodiazepines, cannabis, cocaine, alcohol, and morphine in rats (Miller, 1997; Milanes et al., 1998). Taken together, there is a growing body of literature linking altered stress physiological functioning and drug use vulnerability.

This study was designed to investigate how chronic stress, alcohol consumption, and genotype affect HPA-axis functioning in a sample of rural African American emerging adults residing in the southeastern United States. Beyond the fact that this study includes an at-risk sample that is rarely included in this area of research, we are unaware of any study that includes indicators of environmental stress, alcohol consumption, and genotype as concurrent predictors of HPA functioning. It was hypothesized that increased levels of stress and alcohol consumption would have a larger effect on developing a blunted CAR in comparison to the individual contributions of stress or alcohol alone. The utility of focusing some of our investigation on the CAR is twofold. First, the CAR is a reliable indicator of HPA functioning with hyperactivity associated with the anticipation of acute stress and hypoactivity indicative of a dysregulated HPA in response to chronic exposure to unmitigated stress. Secondly, this biomarker may be a mechanism linking stress dysregulation to drug use vulnerability. Furthermore, we investigated if a person's genotype of the CRH receptor site (CRHR1) would affect basal cortisol levels throughout the day.

Materials and Methods

Participants

Participants ($N = 84$) consisted of African Americans between the ages of 18 and 23 ($\overline{X} = 20.1$, $SD = 1.1$). The majority of the participants were female ($n = 49$, 58.3%), unmarried ($n = 81$, 95.3%), and self-identified as 5th generation in response to immigrant status ($n = 73$, 85.9%). In response to highest level of education obtained, 4.8% had less than a high school education, 33.3% graduated from high school, 50% had some college or technical classes, 10.7% had a college degree, and 1.2% had some professional training. Moreover, 63.1% of the participants were currently unemployed.

Procedures

Participants were recruited to participate in this study after completing their enrollment in the control condition of the adults in the making (AIM) project. This group represents a random sample of African Americans who reside in rural counties in the southeastern U.S. Following informed consent, participants were enrolled in this study and mailed a saliva collection kit. This collection kit contained all the necessary materials and instructions for collecting diurnal samples of salivary cortisol. Participants were instructed to provide six saliva samples in their home using the following schedule: (1) wakeup—before getting out of bed, (2) 30 min post wakeup, (3) 90 min post wakeup, (4) 3:00 PM, (5) 3:30 PM, and (6) 4:30 PM. Samples 1–3 were required as an assessment of the cortisol response to awakening. More specifically, sample 2 represents the cortisol awaking response (CAR). Samples 4–6 provided assessments of the diurnal down-regulation of the HPA-axis through the early evening on a typical day. Participants were instructed not to eat, drink, brush their teeth or smoke cigarettes 30 min prior to sample collection. Compliance with collection protocols were enhanced by having graduate research assistants (GRAs) review the protocol with the participants over the phone prior to starting the saliva collection process. The saliva collection kit also contained collection instructions and a collection diary for the purpose of modeling systematic variation (i.e., time) in our analyses. Female participants were asked to collect home saliva samples outside the menstrual cycle as hormones are uncharacteristically low during this time. Participants were also instructed to freeze the saliva samples in the provided kit immediately upon completion.

During the in-person assessment, two African American GRAs met the participants at a prearranged location (e.g., home) in the participant's community. Participants were oriented to this study and asked to complete a battery of computerized assessments that were randomly administered using Medialab™ v2010 on a laptop PC. Upon completion, the participants completed a 90-day assessment of their alcohol consumption, provided the GRAs with the previously collected saliva samples, and were debriefed. The GRAs transported the frozen saliva samples to the laboratory in sealed coolers filled with freezer bricks in order to prevent a freeze-thaw cycle. Participants were compensated $25 for their participation in this study. This study was approved by the University of Georgia Institutional Review Board.

Measures

Alcohol Consumption

Alcohol consumption over the past 90 days was measured using the Timeline Follow-Back method (Sobell and Sobell, 1992). Participants were provided with a 90-day calendar and asked to provide retrospective estimates of their daily substance use. Several memory aids (i.e., calendar, holidays, key dates, discrete events, and anchor points) were used to enhance recall and alcohol

consumption was reported in standard drinks (i.e., 12 oz beer, 5 oz wine, 1 oz of hard liquor).

CRHR1

Participants provided DNA samples as part of their previous participation in the AIM project. Previously arrayed stock DNA was diluted to 2 ng/ul and robotically dispensed. An informative marker of the corticotropin releasing hormone receptor 1 (CRHR1) was identified from previous studies that investigated depression (Wasserman et al., 2008, 2009) and included a sample of African Americans (Bradley et al., 2008). The samples were amplified using primer probe sets and other reagents from Applied Biosystems (ABI Foster City, CA, USA), then genotyped using an ABI 7900 HT Sequence Detection System using our previously described protocol (Philibert et al., 2009). For the purpose of this study, we will focus on a CRHR1 single nucleotide polymorphism (SNP; dbSNP Marker: rs4792887; Cytogenetic Band: 17q21.31e). This gene encodes a G-protein that binds to neuropeptides of the CRH, a primary regulator of the HPA-Axis. This SNP did not deviate from Hardy-Weinberg equilibrium ($p = 0.21$) and had a 100% call rate in this sample.

Salivary Cortisol

All saliva samples were stored immediately in an ultracold laboratory freezer (−30°C), then shipped overnight frozen with dry-ice pellets to the Middleton Research Biodiagnostics Lab (Madison, WI, USA). On the day of assay, samples were thawed and cortisol was assayed in duplicate using a well-established enzyme-linked immunosorbent assay (ELISA) kit specifically designed for use with saliva (Salimetrics, State College, PA, USA). Mean intra-assay and inter-assay coefficients of variation (CVs) were 3.8% and 7.4%, respectively. Samples were reanalyzed if the CV for the duplicate measurements were >20%. Samples from the same individual were all assayed on the same run. To normalize distributions, extreme values of raw cortisol were winsorized.

Stress

Stress was measured by the Perceived Stress Scale (PSS; Cohen et al., 1983). The PSS is a 14-item self-report measure that assesses an individual's perception of situations in their life that they deem stressful over the past month. The PSS is rated on a 5-point Likert scale ranging from "Never" (0) to "Very Often" (4). Summary scores for the PSS range between 0 and 56; with higher scores indicating more stress. Each item on the PSS assesses one's perceived stress within the last month. In previous research, scores on the PSS demonstrated adequate internal consistency and test—retest reliability. The PSS produced scores with suboptimal but acceptable reliability in this sample (Cronbach's $\alpha = 0.67$).

For descriptive purposes, a stress card sort was used to measure eight potential stressors influencing one's daily life: Family, Friends, Identity, Money, Neighborhood, Race, School, and Work. Each domain included common examples of potential stressors. Participants were instructed to rank order these stressors from the "Most Stressful" to "Least Stressful." Next, they were asked to discuss what about their top stressor was so demanding to deal with.

Analytic Strategy

Hierarchical linear models (HLMs) were use to estimate the trajectory of cortisol throughout the day and accurately account for the inherent nesting of saliva samples ($n = 504$; 6 samples per participant) at level 1 within participants ($n = 84$) at level 2. The dependent variable consisted of six samples of salivary cortisol measured at waking ($\overline{X}_{\text{time}} = 9{:}24$ AM; $TSW = 0$), 30 min after waking ($\overline{X}_{\text{time}} = 9{:}55$ AM, $\overline{X}_{TSW} = 31.51$ min, $SD = 11.02$), 90 min after waking ($\overline{X}_{\text{time}} = 11{:}15$ AM, $\overline{X}_{TSW} = 110.80$ min, $SD = 61.58$), 3:00 PM ($\overline{X} = 3{:}01$ PM, $SD = 22.20$), 3:30 PM ($\overline{X} = 3{:}37$ PM, $SD = 21.13$), and 4:30 PM ($\overline{X} = 4{:}25$ PM, $SD = 18.45$). At level 1, two predictors of cortisol level accounted for the trajectory of cortisol throughout the day. First, time since waking ($\beta_{1\ TSW}$) was modeled in min and used to capture the passage of time associated with the collection of six saliva samples from waking to 4:30 PM. Assuming a diurnal slope of cortisol throughout the day, the slope of TSW would reflect a curvilinear decrease in cortisol from waking to 4:30 PM. However, it is likely that the participants would experience a peak cortisol response approximately 30 min after waking up. Therefore, we included a second variable to capture the (CAR; $\beta_{2\ CAR}$), which was modeled using a dummy variable that was coded 1 for Sample #2 (30 min after waking) and 0 for the remaining samples. Four samples were excluded from the HLM model since their sample was collected more than 50 min from waking. The intercept (β_0) therefore represents cortisol level at baseline.

An advantage of HLM is that these predictors of level 1 cortisol functioning can become outcomes of interest using a slopes-as-outcomes approach (Snijders and Bosker, 1999). Individual differences in these terms were modeled to allow for each individual to have different cortisol levels at baseline (U_0); different levels of cortisol down-regulation across the day (U_1); or different rises in cortisol in response to waking (U_2). The equations below illustrate that the CRHR1 genotype was entered as a main effect on cortisol level at baseline. Furthermore, perceived stress ($\gamma_{21\ PSS}$) and standard drinks consumed over the past 90 days ($\gamma_{22\ EtOH}$) were entered as main effects on CAR.

$$\text{Level 1 (within-individual) Cortisol} = \beta_0 + \beta_{1\ TSW} + \beta_{2\ CAR} + \text{r}$$

$$\text{Level 2 (between individual)} \quad \beta_0 = \gamma_{00} + \gamma_{01\ CRHR1} + U_0$$

$$\beta_{1\ TSW} = \gamma_{10} + U_1$$

$$\beta_{2\ CAR} = \gamma_{20} + \gamma_{21\ PSS} + \gamma_{22\ EtOH} + U_2$$

Results

Daily Stressors

Work (20.2%), Money (15.9%), and Neighborhood (15.5%) stress were identified as the top three stressors that were influencing one's daily life. Work related stressors included problems finding a job, being laid off, and experiences of racism, discrimination, or some other type of unfair treatment. Money related stressors coalesced around difficulties making enough money, paying bills, and providing for one's family. Finally, neighborhood stress was largely associated with living in poverty, fear for one's safety, and exposure to drugs, crime, and violence.

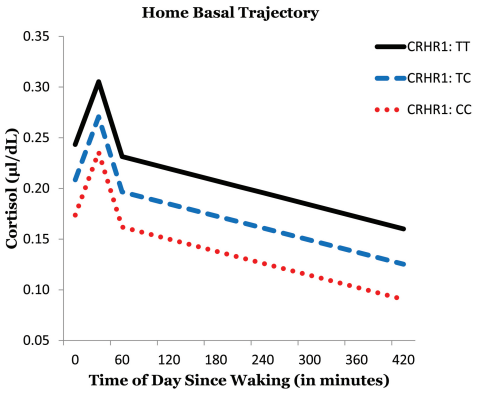

FIGURE 1 | Effects of CRHR1 genotype on basal cortisol levels.

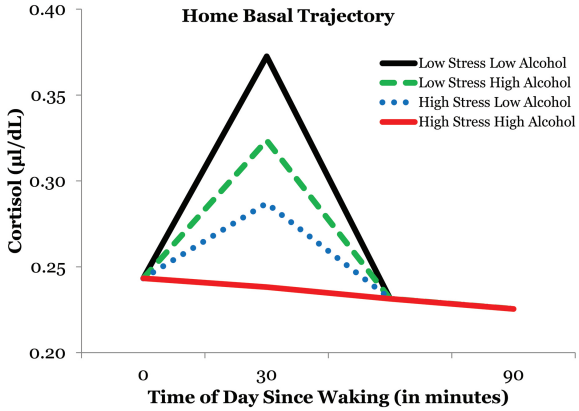

FIGURE 2 | The effects of alcohol consumption and perceived stress on the cortisol awaking response.

Stress and Alcohol Use

All of the participants reported that they experienced stress during the past month. More specifically, the scores on the PSS ranged from 8 to 40 with an average score of 24.7 (SD = 6.3). Additionally, 63.5% of the participants reported alcohol consumption during the past 90-days. On average, the participants reporting consuming approximately 14 drinks (SD = 28.7) during this reporting period (range: 0–140).

Basal Cortisol Levels

TSW represented a significant linear slope from waking until the last sample was collected [$\beta_1 = -0.0003, t(70) = -5.71, p < 0.001$]. On average, cortisol levels decreased throughout the day. CAR also represented a significant linear slope to the model [$\beta_2 = 0.07$, $t(70) = 3.56$, $p = 0.001$]. This positive relationship indicated that cortisol levels increased from waking to approximately 30 min after waking. After accounting for the diurnal rhythm and the CAR, 70.6% of the total variance in cortisol was found to be a function of systematic individual differences [$\chi^2(52) = 225.56$, $p < 0.001$] and the remaining 29.4% of the variance in cortisol was derived from moment to moment fluctuations in cortisol beyond the diurnal rhythm or CAR. This suggests that rural African American emerging adults have moderately systematic salivary cortisol levels.

Effect of CRHR1 Genotype on Cortisol Level at Baseline

This sample had the following genotype frequencies: CC (38.8%), CT (40%), TT (7.1%); with 7.1% missing data. We tested to see if some of the variability in basal cortisol levels could be accounted for by an individual's genotype. The T allele was coded to indicate greater dose: TT = 2, TC = 1, CC = 0. The CRHR1 SNP (rs4792887) had a significant linear relationship with cortisol level at baseline [$\gamma_{01} = 0.048$, $t(69) = 2.21$, $p = 0.031$]. Rural African American emerging adults with the TT genotype of the CRHR1 gene tended to produce the most basal cortisol throughout the day (**Figure 1**). Furthermore, participants with the CC genotype produced the least amount of basal cortisol. This provided some initial evidence for an additive relationship, with each additional T allele associated with greater basal cortisol output.

Alcohol Consumption and Perceived Stress Differences in the CAR

We tested to see if some of the variability in CAR could be accounted for by perceived stress over the past month and standard drinks consumed over the past 90 days. Perceived stress had a significant inverse relationship with CAR [$\gamma_{21} = -0.007$, $t(68) = -2.33$, $p = 0.023$]. More specifically, rural African American emerging adults had a blunted CAR when experiencing higher levels of perceived stress. Furthermore, alcohol consumption also had a significant independent inverse relationship with CAR [$\gamma_{22} = -0.003$, $t(68) = -2.13$, $p = 0.037$] such that the CAR was blunted in individuals who consumed higher amounts of alcoholic beverages. Moreover, the CAR was essentially non-existent in participants who reported higher levels of stress and alcohol consumption (**Figure 2**). The interaction between reported stress and alcohol consumption was not a predictor of CAR suggesting additive effects of perceived stress and alcohol consumption. Additionally, there wasn't a gender effect in predicting CAR.

Discussion

The CAR has been identified as a reliable indicator of HPA-axis functioning (Chida and Steptoe, 2009; Gonzalez et al., 2009; Thorn et al., 2009). In this study, rural African American emerging adults exhibited a blunted CAR when reporting higher levels of stress or alcohol consumption. Moreover, these effects were additive, such that the CAR was obliterated for participants who reported both the experience of higher stress and increased alcohol consumption. While stress was only measured quantitatively over the past month, this outcome is consistent with previous studies that found a hypoactive CAR to be linked to chronic stress, fatigue, burnout, and hopelessness (Chida and Steptoe, 2009; Fries et al., 2009). This is further substantiated by the types of chronic stressors that were identified by the stress card sort. Furthermore, previous research linked alcohol abuse with hyperactive HPA activity (Adinoff et al., 2003). It is our hypothesis, that alcohol is being consumed as a negative coping strategy for addressing unmitigated stressors that this community endures on a daily basis. The long-term effect of heavy alcohol use

will primarily mirror that of the stress model where acute alcohol consumption aimed at coping with daily life stress will initially lead to a hyperactive HPA-axis. However, the body's chronic exposure to increased levels of alcohol will consequentially cause wear-and-tear on this regulatory system and ultimately result in a hypoactive HPA-axis due to allostatic load (Lovallo et al., 2000; Koob and Le Moal, 2001; McEwen, 2004; Lovallo, 2006). This is consistent with our finding that an increased level of alcohol consumption was linked to a blunted CAR.

Additionally, this study investigated the role that genotype—specifically CRHR1—might have in the production of basal cortisol. African American emerging adults with a CC genotype in the CRHR1 SNP (rs4792887) showed the lowest production of basal cortisol while participants with the TT genotype showed the highest levels of basal cortisol. Previous studies have identified the T allele as a risk factor in depression and suicidality (Wasserman et al., 2008, 2009). While exploratory, the data suggests an additive effect where the T allele in this particular SNP may be a risk factor associated with psychological distress which may lead to the production of higher basal cortisol. This is of particular importance given the growing body of literature linking dysregulated HPA-functioning with poor health outcomes (Fries et al., 2009).

It is important to note that experiences of stress related to work (i.e., finding a job, being laid off, experiences of racism, and discrimination), money (i.e., difficulty paying bills and providing for one's family), and neighborhood disorganization (i.e., living in poverty, fear for one's safety, and exposure to drugs, crime, and violence) were the top three chronic stressors identified by this sample of rurally situated African American emerging adults. This data is consistent with national trends showing African Americans (16.0%) experiencing an unemployment rate that nearly doubles that of their European American counterparts (8.7%; U.S. Department of Labor, 2011). More specifically, this cohort reported an unemployment rate of 63.1%. While it remains unclear if this cluster of chronic stressors is a function of race, rural status, age, and/or post-recession outcomes, there is little doubt that this community is exposed to unmitigated stressors that may have a deleterious effect on their long-term health outcomes. This "wear and tear" already appears evident within these emerging adults' HPA axis.

While this study provides some unique data illustrating a relationship between stress, alcohol consumption, genotype, and HPA dysregulation with a rarely studied at-risk population, it is not without limitations. First, this study could benefit from a more nuanced assessment of environmental and neighborhood characteristics that serve as chronic stressors for this population. While the card-sort and assessment of perceived stress generated interesting data to substantiate the need for further investigation, more objective assessments utilizing geographic information system (GIS) mapping could accurately characterize drug availability, potential sources of chronic stress, and risk factors associated with drug use in the neighborhoods that the research participants currently reside in. Secondly, basal cortisol samples were collected at six points across the day; with the last sample being collected at approximately 4:30 pm. Given the termination of sample collection in the early evening, we are unable to model continued decreases in cortisol levels through bedtime. While this was done to time match these samples with a laboratory-based stress paradigm that took place in a second wave of this study, not reported here, the basal data utilized in the HLM model illustrates a clear reduction in cortisol across time, but doesn't include the lowest point prior to sleep. Additionally, salivary cortisol was only collected across 1 day and did not include an objective assessment of time reporting. Therefore, there could be some error in the self-reporting of time in addition to the inability to make conclusions regarding the trait levels of basal cortisol due to the potential of day-to-day variations. Of note, we did not assess which participants from this community sample were currently enrolled in school. As a result, we could not assess what percentage of the unemployment rate was a function of being enrolled in school—regardless of work and money being identified as the top two stressors influencing their daily lives. Finally, this sample consisted of African American emerging adults residing in rural counties. Generalizing these findings to urban/suburban communities and/or settings should only be done with caution until such data becomes available. Given these limitations, the data provides some novel insights into the relationship between alcohol consumption, perceived stress, and CRHR1 genotype with the HPA-axis in a rarely studied sample of rural African Americans.

Future research could benefit from a prospective longitudinal research design that can ascertain if stress dysregulation leads to drug-use vulnerability, if drug-use accelerates the process of stress dysregulation, or both. Understanding the directionality of this relationship could lead to the identification of robust targets of intervention and prevention. This area of research could also be advanced by utilizing objective GIS data in characterizing neighborhood environments (Theall et al., 2012, 2013). Additionally, this body of literature could be enhanced by integrating decision-making variables, such as delayed discounting or alcohol demand, as proximal mechanisms that connect stress dysregulation to actual choices to drink or not to drink (MacKillop et al., 2010, 2011). Finally, the inclusion of objective measures of ethnicity and culture—in addition to other at-risk populations—could shed some light on within-group and between-group drug-related health disparities that disproportionately affect marginalized communities. In conclusion, the African American community disproportionately experiences poor health outcomes and further investigation is needed in this area to uncover and refine the mechanisms that can explain and eliminate this growing public health problem.

Author Contributions

All authors contributed in a significant way to the manuscript and have read and approved the final manuscript.

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FRAS1-related extracellular matrix 3 (*FREM3*) single-nucleotide polymorphism effects on gene expression, amygdala reactivity and perceptual processing speed: An accelerated aging pathway of depression risk

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The A allele of the FRAS1-related extracellular matrix protein 3 (*FREM3*) rs7676614 single nucleotide polymorphism (SNP) was linked to major depressive disorder (MDD) in an early genome-wide association study (GWAS), and to symptoms of psychomotor retardation in a follow-up investigation. In line with significant overlap between age- and depression-related molecular pathways, parallel work has shown that *FREM3* expression in postmortem human brain decreases with age. Here, we probe the effect of rs7676614 on amygdala reactivity and perceptual processing speed, both of which are altered in depression and aging. Amygdala reactivity was assessed using a face-matching BOLD fMRI paradigm in 365 Caucasian participants in the Duke Neurogenetics Study (DNS) (192 women, mean age 19.7 $\pm$ 1.2). Perceptual processing speed was indexed by reaction times in the same task and the Trail Making Test (TMT). The effect of rs7676614 on *FREM3* mRNA brain expression levels was probed in a postmortem cohort of 169 Caucasian individuals (44 women, mean age 50.8 $\pm$ 14.9). The A allele of rs7676614 was associated with blunted amygdala reactivity to faces, slower reaction times in the face-matching condition ($p < 0.04$), as well as marginally slower performance on TMT Part B ($p = 0.056$). In the postmortem cohort, the T allele of rs6537170 (proxy for the rs7676614 A allele), was associated with trend-level reductions in gene expression in Brodmann areas 11 and 47 ($p = 0.066$), reminiscent of patterns characteristic of older age. The low-expressing allele of another *FREM3* SNP (rs1391187) was similarly associated with reduced amygdala reactivity and slower TMT Part B speed, in addition to reduced BA47 activity and extraversion ($p < 0.05$). Together, these results suggest common genetic variation associated with reduced *FREM3* expression may confer risk for a subtype of depression characterized by reduced reactivity to environmental stimuli and slower perceptual processing speed, possibly suggestive of accelerated aging.

Keywords: depression, aging, amygdala, Frem3, processing speed

Introduction

Despite increasing sample sizes, case-control genome-wide association studies (GWAS) of major depressive disorder (MDD) have yielded inconclusive results, with few single nucleotide polymorphisms (SNPs) surviving the commonly accepted genome-wide significance threshold of $p = 5 \times 10^{-8}$ and even fewer withstanding the test of a full replication in an independent sample (Wray et al., 2012; Major Depressive Disorder Working Group of the Psychiatric GWAS Consortium et al., 2013). The relative lack of success of these studies has been at least partially attributed to the heterogeneity inherent to the MDD diagnostic category (Major Depressive Disorder Working Group of the Psychiatric GWAS Consortium et al., 2013). Yet, despite the recognition in the literature of different MDD subtypes with presumably different pathophysiologies (Kessing, 2007), few genetic association studies take this heterogeneity into account, and those that do, have been inadequately powered to detect subtype-specific associations (Major Depressive Disorder Working Group of the Psychiatric GWAS Consortium et al., 2013).

Despite these initial set-backs, several follow-up investigations have resulted in partial replications or have linked suggestive SNP "hits" to specific symptom dimensions or biological endophenotypes, though not necessarily to the overall syndrome (Sullivan et al., 2009; Rietschel et al., 2010; Kohli et al., 2011; Kuehner et al., 2011; Li et al., 2013; Schuhmacher et al., 2013). In light of the immense diagnostic, and presumably biological heterogeneity of MDD, such partial replications and mechanistic follow-up studies may in fact inform an improved understanding of the etiology of specific subtypes of MDD as well as help facilitate the delineation of distinct biological pathways of risk.

A SNP (rs7676614) within the human FRAS1-related extracellular matrix protein 3, encoded by the *FREM3* gene, was among the top hits in an early GWAS of MDD, where the major A allele was associated with nominally higher risk relative to the minor G allele ($p = 9.52 \times 10^{-5}$ meta-analyzed across two independent samples) (Muglia et al., 2010). Importantly, while this SNP was not associated with MDD diagnosis in a follow-up study, it was specifically and strongly linked to symptoms of psychomotor retardation (Shi et al., 2012). Psychomotor symptoms in depression can range from retardation to agitation and have been proposed to constitute an important source of disorder heterogeneity (Sobin and Sackeim, 1997; Leventhal et al., 2008; Schrijvers et al., 2008). Thus, the association between rs7676614 and symptoms of retardation may not only explain the lack of a stronger link between the SNP and overall MDD diagnosis, but also offer mechanistic clues as to the specific biological pathway through which this SNP may increase MDD risk.

A largely independent line of research drawing on human postmortem gene expression data has demonstrated a significant overlap between molecular pathways implicated in depression and normal aging (Wolkowitz et al., 2010; Sibille, 2013). Specifically, it has been proposed that MDD or risk thereof may stem at least partially from molecular processes resulting in gene expression patterns reminiscent of premature, or accelerated, aging of the brain (Douillard-Guilloux et al., 2013; Sibille, 2013). Intriguingly, *FREM3* is among the genes whose expression declines as a function of normal aging (Glorioso et al., 2011). This steady decline has been shown to occur in the amygdala and the anterior cingulate cortex (ACC), both of which have been extensively implicated in the pathophysiology of depression (Davidson et al., 2002). While this prior work has used a relatively small cohort enriched for individuals over the age of 50, thus possibly not fully capturing age-related changes in expression pattern occurring throughout the entire adult lifespan, it strongly suggests *FREM3* may contribute to age-related pathways of depression risk. Furthermore, the retardation of psychomotor function, in addition to being a symptom of a subtype of depression, is also a concomitant of older age (Seidler et al., 2010). Taken together, these data raise the intriguing possibility that the A allele of the *FREM3* rs7676614 polymorphism may predispose to depression via an accelerated brain aging pathway, or, alternatively, the minor G allele may be associated with relative resilience to, or slowing of, the normal changes associated with aging.

Depression and aging are both associated with profound changes in cognitive and affective processing in the brain (Milham et al., 2002; Whalen et al., 2002; Fitzgerald et al., 2008; Disner et al., 2011; Kehoe et al., 2013; Kennedy et al., 2015). A large body of work utilizing functional magnetic resonance imaging (fMRI) in patients with MDD has revealed dysregulation within a corticolimbic circuitry involved in the perception and regulation of emotion and affect, within which the amygdala serves as a hub. The majority of these studies demonstrate relative amygdala hyperactivity in response to negative stimuli (Whalen et al., 2002; Victor et al., 2010; Yang et al., 2010). However, a notable minority of studies has associated depression (Thomas et al., 2001) or depression risk (Wolfensberger et al., 2008) with relatively blunted amygdala response to threat, suggesting there may be a depression subtype associated with reduced reactivity to environmental stimuli. Notably, amygdala reactivity is also reduced in healthy older adults (Fischer et al., 2005, 2010; Tessitore et al., 2005). Consistent with molecular pathology findings in depression (Douillard-Guilloux et al., 2013), it is possible that a premature forward shift along this normal age-related trajectory of corticolimbic function may contribute to risk of depression or one of its subtypes. Conversely, factors that slow progression along this trajectory may confer resilience.

In the current study, we leveraged data from the ongoing Duke Neurogenetics Study (DNS) and a postmortem cohort, to test the hypothesis that rs7676614 would modulate risk of depression via accelerating normal age-related changes in neuropsychological function and gene expression patterns. Specifically, we hypothesized that, regardless of current depressive symptomatology, the A allele would bias amygdala reactivity, perceptual processing speed, and gene expression toward a pattern reminiscent of older age. We further sought to confirm the effect of age on *FREM3* gene expression in the current postmortem cohort, which is better powered to detect trajectories of gene expression change across the entire adult lifespan than previous ones (Erraji-Benchekroun et al., 2005; Glorioso et al., 2011), while also extending the results to the

orbitofrontal and ventrolateral prefrontal cortices (Brodmann areas 11 and 47). Finally, we explored the possible involvement of *FREM3* in depression risk by linking additional functional variation in the same gene (rs1391187) to behaviorally relevant differences in *in vivo* brain function.

Methods

Participants

Neuroimaging and genetic data were derived from 365 non-Hispanic Caucasian participants (192 women, mean age 19.76 ± 1.23) who had successfully completed the ongoing Duke Neurogenetics Study (DNS) and whose BOLD fMRI data survived a stringent multi-level quality control procedure (Supplementary Methods). The DNS assesses a range of behavioral and biological traits among young adult, university students. All participants provided informed consent in accordance with Duke University guidelines, and were in good general health. All participants were free of the following exclusionary criteria, as determined by self-report: (1) medical diagnoses of cancer, stroke, diabetes requiring insulin treatment, chronic kidney or liver disease, or lifetime history of psychotic symptoms; (2) use of psychotropic, glucocorticoid, or hypolipidemic medication; and (3) conditions affecting cerebral blood flow and metabolism (e.g., hypertension). Participants were also screened for DSM-IV Axis I and select Axis II diagnoses (Antisocial and Borderline Personality Disorder) using the eMINI (Sheehan et al., 1998). Current or lifetime diagnosis of a disorder was not exclusionary. However, all participants were free of psychotropic medication, including recreational drugs, at the time of study participation. Notably, controlling for current or past DSM-IV diagnosis did not significantly alter our results. Thus, we report results from models not including diagnosis as a covariate. Detailed diagnostic information can be found in Supplementary Table 1.

BOLD fMRI Paradigm

Our amygdala reactivity paradigm has been described in detail previously (Carré et al., 2013). Briefly, the amygdala reactivity paradigm consists of 4 blocks of a face-processing task interleaved with 5 blocks of a sensorimotor control task. During task blocks, participants are simultaneously presented with three faces (with neutral, angry, fearful, or surprised expressions) and are asked to indicate which one of two faces shown in the bottom of the screen is identical to a target face shown on top. During control blocks, participants perform an analogous task with simple geometric shapes. Here, we focused on amygdala reactivity in the contrast of face blocks vs. control blocks (i.e., All Faces > Shapes). To ensure greater convergence between our *in vivo* neuroimaging and our post-mortem data analyses, we further probed the effect of genotype on activity in BA11/47, corresponding to regions of the orbitofrontal cortex (OFC) and ventrolateral prefrontal cortex (vlPFC). In a follow-up exploratory analysis we probed genotype effects on threat-specific amygdala reactivity in the Anger + Fear > Shapes condition.

Perceptual Processing Speed

Perceptual processing speed was assessed using reaction times (RT) recorded during the face and shape-matching task. Notably, even though the stimuli remained on the screen for a fixed amount of time to ensure uniform perceptual exposure, participants were instructed to match the stimuli as quickly as they can, while maintaining accuracy. RTs were recorded in all trials. Accuracy was close to ceiling throughout the task, but was slightly higher in the Faces (98.7% ± 0.05), relative to the Shapes (98.0% ± 0.05) condition ($p < 0.001$). Only RTs from correct trials in both conditions were used in analyses.

To further probe perceptual and cognitive processing speed we used the Trail Making Test (TMT, Bowie and Harvey, 2006). This test is designed to measure processing speed, cognitive flexibility as well as visuomotor skills. Part A of the TMT (Trails A) asks participants to connect encircled numbers 1 through 25 sequentially on a piece of paper. In Part B of the TMT (Trails B) participants connect encircled numbers and letters, in numerical and alphabetical order, respectively, while alternating between numbers and letters. Numbers and letters are distributed on the paper in a semi-random fashion that allows them to be connected without drawing overlapping lines. The primary outcome of interest in both Parts A and B of this test is the overall time it takes for participants to complete the task. Trails A is believed to gauge visual search and motor speed skills, while Trails B is believed to tap into higher-level cognitive skills, such as mental flexibility. Outliers greater than 60 s on Trails A ($n = 2$) or 90 s ($n = 3$) on Trails B were removed from analyses. Number of errors was controlled for alongside gender and age in all analyses involving the TMT.

Self-report and Behavioral Measures

Recent and early life stress were assessed using a modified version of the Life Events Scale for Students (LESS, Nikolova et al., 2012) and the Childhood Trauma Questionnaire (CTQ, Bernstein et al., 2002), respectively. Current depression and anxiety were assessed using the Center for Epidemiological Studies-Depression (CES-D, Radloff, 1977) scale and the short form of the Mood and Anxiety Symptom Questionnaire (MASQ, Watson et al., 1995). To assess the effects of genotype on personality traits which may predispose to or protect against depression, we analyzed response data on the NEO personality inventory (Costa and McCrae, 1992). We focused specifically on the neuroticism (NEON) and extraversion (NEOE) factors, which have been most extensively mapped onto specific neurobiological substrates (Wright et al., 2006; Aghajani et al., 2014), as well as differential risk or resilience for psychopathology (Campbell-Sills et al., 2006; Bienvenu et al., 2007; Lamers et al., 2012). The Wechsler Abbreviated Scale of Intelligence™ (WASI™, Wechsler, 1999) was administered to assess general intelligence, which was used as a covariate in analyses involving the TMT.

Genotyping

Genotyping of the *in vivo* cohort was facilitated through 23andMe (23andMe, Inc., Mountain View, CA). Genomic DNA from all participants was isolated from buccal cells and leukocytes derived from Oragene DNA self-collection kits (DNA Genotek,

Inc., Kanata, Ontario, Canada) customized for 23andMe. DNA extraction and genotyping were performed at the National Genetics Institute (NGI), a CLIA-certified clinical laboratory and subsidiary of Laboratory Corporation of America. The Illumina Omni Express Plus chip (Illumina, Inc., San Diego, CA, USA) and a custom array containing an additional ∼300,000 SNPs were used to provide genome-wide data (Eriksson et al., 2010; Do et al., 2011; Tung et al., 2011). The Illumina Omni Express Plus chip included the rs7676614 SNP. In our final sample, genotype frequencies for rs7676614 did not deviate from Hardy-Weinberg equilibrium (169 A homozygotes, 166 AG heterozygotes, 30 G homozygotes, $\chi^2 = 1.49$; $p = 0.22$). Furthermore, the allele frequencies in the current sample (A allele: 0.31, G allele: 0.69) were similar to those reported for populations of European ancestry (current sample: A allele frequency 0.31, G allele: 0.69; reference sample from 1000 Genomes, A allele: 0.35, G allele: 0.65).

Postmortem Gene Expression

The postmortem cohort has been described in detail previously (Seney et al., 2013). Briefly, data were derived from 211 individuals (44 women, mean age 50.76 ± 14.91, range 16–96) with no history of neuropsychiatric illness. After consent from next-of-kin was obtained, postmortem brains were collected in the Allegheny County Coroner's Office (Pittsburgh, PA) using procedures approved by University of Pittsburgh's Institutional Review Board and Committee for Oversight of Research Involving the Dead. The absence of psychiatric DSM-IV diagnosis was determined based on psychopathology, medical and social histories, as well as history of substance abuse. Individuals were also screened for the absence of neurodegenerative disorders by neuro-pathological examination.

Total RNA was extracted from frozen samples using TRIzol® (Invitrogen Life Technologies, Carlsbad, CA, USA) following manufacturer's protocol (see Supplementary Methods). Technical parameters of all RNA samples were within the range of desired RNA quality for large-scale gene expression studies (RNA integrity number: 8.02 ± 0.74, RNA ratio: 1.53 ± 0.35). RNA samples were processed for microarray by the Gene Expression & Genotyping Core Facility at Case Western Reserve University. Briefly, with 150 ng of total RNA, cDNA was synthesized by Ovation PicoSL WTA System V2 and labeled with Encore Biotin Module (both from NuGEN Technologies, San Carlos, CA). 2.5 μg of cDNA was hybridized on Affymetrix® Human Gene 1.1 ST arrays (Affymetrix, Santa Clara, CA), covering over 30,000 coding transcripts. Array hybridization, washing, and staining were conducted on GeneTitan® (Affymetrix) according to the manufacturer's protocol. Gene expression values were extracted via Expression Console build 1.2.1.20 using RMA method and quantile-normalization to eliminate batch effects. Gene expression probes were processed in gene-level and converted to log2 scale to perform further analysis. One coding transcript corresponding to the *FREM3* gene was represented on the array. Analyses were restricted to Caucasian individuals, whose samples passed quality control ($n = 169$, 44 women, mean age 50.8 ± 14.9; see also Supplementary Methods).

Total DNA was extracted from fresh frozen brain samples with Qiagen DNA mini kit following manufacturer's protocol. Genotype calls were generated using Affymetrix Genotyping Console version 4.1.3 by a Birdseed v2 algorithm, which uses EM to drive maximum likelihood fit of two dimensional Gaussian mixture models. As the *FREM3* rs7676614 SNP was not available on the Affymetrix SNP Array 6.0, a perfect proxy SNP, rs6537170 ($R^2 = 1.00$, $D' = 1.00$), was identified using data from the 1000 Genomes project (Genomes Project et al., 2012) accessed via the online SNAP Proxy Search Tool of the Broad Institute (Johnson et al., 2008). This proxy SNP was used in all analyses involving postmortem samples. We probed the effect of rs6537170 on *FREM3* mRNA levels in samples derived from both BA11 and BA47 in 169 Caucasian individuals. An additional 57 SNPs within or near the *FREM3* gene (50 kb up- or downstream) were examined for association with *FREM3* expression (Supplementary Table 2). To further explore the involvement of *FREM3* in depression-related phenotypes, we selected the SNP with the strongest impact on *FREM3* expression (rs1391187) and evaluated its effect (via proxy SNP rs1909022, $R^2 = 1.00$, $D' = 1.00$, 1000 Genomes) on the same *in vivo* neural and behavioral phenotypes tested for rs7676614.

Statistical Analyses

Three sets of models were tested for all *in vivo* phenotypic associations: additive, minor allele dominant, and minor allele recessive. In the additive model, linear regressions using number of major alleles as a predictor of amygdala reactivity or perceptual processing time were conducted in IBM SPSS Statistics 21 (IBM, Armonk, NY). In the dominant model, ANOVAs using genotype (minor allele carrier vs. major allele homozygote, or major allele carrier vs. minor allele homozygote) predicting outcomes were used. In the postmortem cohort, a general power function model was used to assess the effects of age on gene expression scores residualized for potential confounding covariates (e.g., postmortem interval, pH, RNA integrity number, race, and gender). This model was adopted in order to allow for the detection of non-linear age-related trajectories. The effects of genotype on expression was tested using *FREM3* expression scores residualized for those potential confounds in addition to estimated non-linear age effect, ethnic background (Price et al., 2006), and 16 principal components derived from transcriptome-wide analysis of gene expression data (Liang et al., 2013). Mediation analyses in the *in vivo* cohort were conducted using Models 4 and 6 of the PROCESS macro in SPSS (Hayes, 2013). Bootstrapped bias-corrected confidence intervals (CIs) for each indirect effect were generated using 5000 bootstrapping iterations.

Results

Main Effect of Task and Demographics

Consistent with prior research (Nikolova and Hariri, 2012; Swartz et al., 2015), our task resulted in significant amygdala reactivity bilaterally for the Faces > Shapes contrast (**Figure 1A**). Further analyses revealed significant activity in BA11 and BA47 (**Figure 1B**). Partially confirming prior work (Nikolova et al.,

2012), we found that men had greater amygdala reactivity than women in the left [$t_{(363)} = 2.54$, $p = 0.012$], but not the right hemisphere [$t_{(363)} = 1.30$, $p = 0.193$]. When gender was accounted for, amygdala reactivity was further modulated by age, such that older participants showed a trend towards lower amygdala reactivity (left hemisphere: $b = -0.087$, $p = 0.096$; right hemisphere: $b = -0.090$, $p = 0.085$). There were no differences in age, gender composition and estimated IQ among genotype groups (**Table 1**). However, in light of the effects of gender and age on amygdala reactivity, all analyses were conducted with and without gender and age as covariates.

Genetic Effects on Amygdala Reactivity

FREM3 rs7676614 genotype modulated amygdala response, such that increasing number of major (A) alleles was associated with significantly decreased amygdala reactivity in the right ($b = -0.109$, $p = 0.038$), and marginally decreased reactivity in the left ($b = -0.101$, $p = 0.055$) hemisphere (**Figures 2A,C**). The effect was significant bilaterally when covarying for gender and age (right hemisphere: $b = -0.116$, $p = 0.026$; left hemisphere: $b = -0.110$, $p = 0.035$). When a G allele dominant model was tested, individuals homozygous for the risk A allele were found to have relatively reduced amygdala reactivity for the All Faces > Shapes contrast, when compared to carriers of the G allele. The effect was stronger on the right side [left hemisphere: $F_{(1, 363)} = 3.06$, $p = 0.081$; right hemisphere: $F_{(1, 363)} = 4.46$, $p = 0.035$; **Figures 2B,D**]. The significance of these results did not change when gender and age were controlled for [left hemisphere: $F_{(1, 361)} = 3.85$, $p = 0.051$; right hemisphere: $F_{(1, 361)} = 5.20$, $p = 0.023$]. Notably, no significant effect of genotype emerged in threat-specific contrasts ($p > 0.11$), suggesting a broader hypo-reactivity of the amygdala to environmental salience rather than a threat-specific blunting of amygdala response. Consistent with this conceptualization, neither current, nor early life stress interacted with *FREM3* genotype to predict amygdala response ($p > 0.12$). Importantly, there were no differences in self-reported early or recent stress between genotype groups ($p > 0.10$).

An exploratory whole-brain analysis revealed no effect of *FREM3* rs7676614 genotype on activity in any other brain region. Further region of interest analyses revealed no effect on activity in BA11 and BA47 ($p > 0.10$).

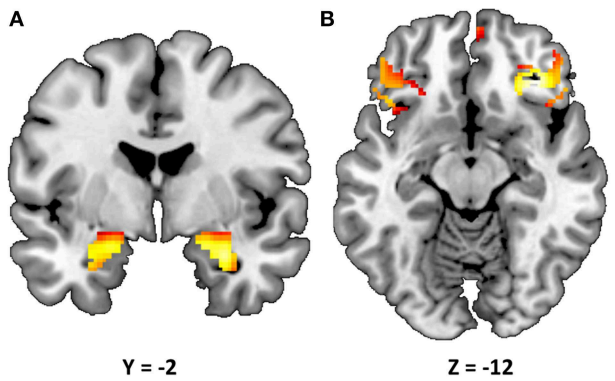

FIGURE 1 | Statistical parametric map illustrating mean bilateral activity for the Faces > Shapes contrast. Activation clusters are presented for **(A)** the amygdala (peak voxel activation in the left hemisphere: $x = -22$, $y = -6$, $z = -18$, $t = 28.55$, $p < 0.000001$, $kE = 179$; right hemisphere: $x = 28$, $y = -4$, $z = -20$, $t = 32.82$, $p < 0.000001$, $kE = 199$) and **(B)** Brodmann areas 11 and 47 (peak voxel activation in the left hemisphere: $x = 58$, $y = 28$, $z = 0$, $t = 15.60$, $p < 0.000001$, $kE = 143$; right hemisphere: $x = -52$, $y = 40$, $z = -2$, $t = 14.47$, $p < 0.000001$, $kE = 24$). The clusters are overlaid onto canonical structural brain images in the coronal plane ($y = -2$) and axial planes ($z = -12$), respectively.

Genetic Effects on Processing Speed Reaction Times

In addition to its effect on amygdala response, *FREM3* genotype was found to modulate processing speed as indexed by behavioral measures collected during the BOLD fMRI paradigm. Specifically, increasing number of A alleles was associated with slower RTs in the Faces ($b = -0.107$, $p = 0.035$; controlling for gender and age: $b = -0.109$, $p = 0.032$), but not in the Shapes ($p > 0.2$; **Figures 3A,C**) condition. Similarly, G allele carriers had faster RTs relative to A homozygotes in the Faces [A homozygotes: 1.22 ± 0.28 s; G carriers: 1.15 ± 0.27 s; $F_{(1, 363)} = 4.89$, $p = 0.028$; controlling for gender and age: $F_{(1, 361)} = 5.00$, $p = 0.026$], but not in the Shapes blocks [A

TABLE 1 | Summary of demographic characteristics and estimated IQ represented as a function of *FREM3* rs7676614 genotype.

	FREM3 rs7676614			*p*	*p* (additive)
	GG	AG	AA		
n	30	166	169	‘	‘
Male/Female	14/16	76/90	83/86	0.827	‘
Age (mean ± SD)	19.83 ± 1.26	19.83 ± 1.25	19.67 ± 1.20	0.499	0.278
Estimated IQ	125.33 ± 6.47	123.70 ± 7.12	123.26 ± 7.70	0.358	0.200
CTQ	31.13 ± 6.64	31.09 ± 6.70	31.85 ± 8.23	0.632	0.390
LESS	3.97 ± 2.39	3.98 ± 2.74	4.49 ± 3.26	0.270	0.138
NEO-N	77.83 ± 23.64	82.58 ± 20.59	82.50 ± 24.10	0.549	0.490
NEO-E	125.30 ± 20.08	120.81 ± 21.40	122.09 ± 20.92	0.543	0.839

The table includes p-values from both a mean comparison (ANOVA) and an additive (regression) model.

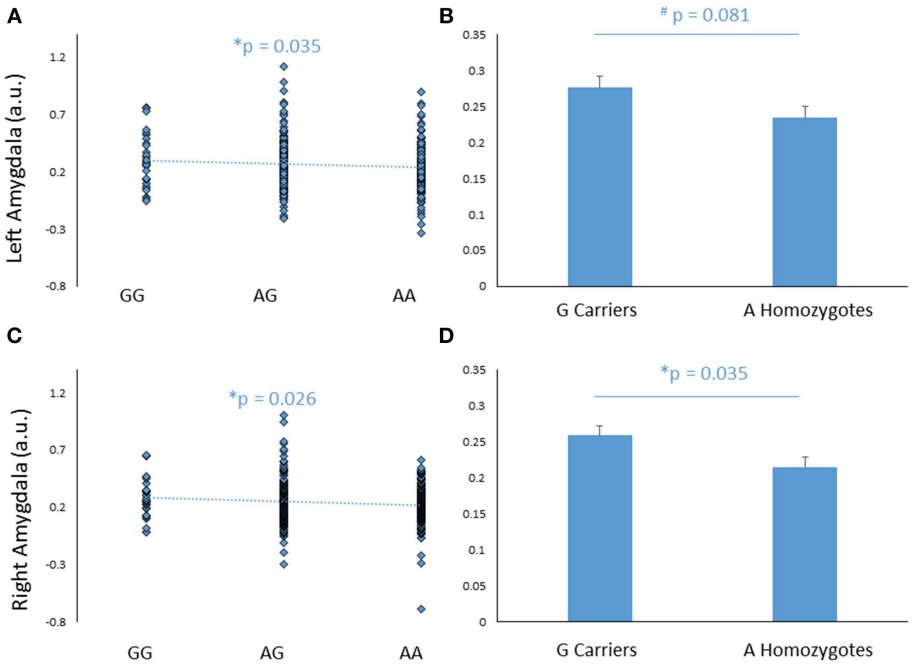

FIGURE 2 | Effects of *FREM3* rs7676614 genotype on amygdala reactivity. Increasing number of A alleles was associated with decreased amygdala reactivity in the left **(A)** and right **(C)** hemisphere. In a G allele dominant model, A homozygotes showed a trend toward decreased amygdala reactivity in the left hemisphere **(B)** and significantly decreased amygdala reactivity than G carriers in the right hemisphere **(D)**. Error bars represent standard error of the mean. $^{*}p < 0.05$, $^{\#}p < 0.10$, a.u. = arbitrary units.

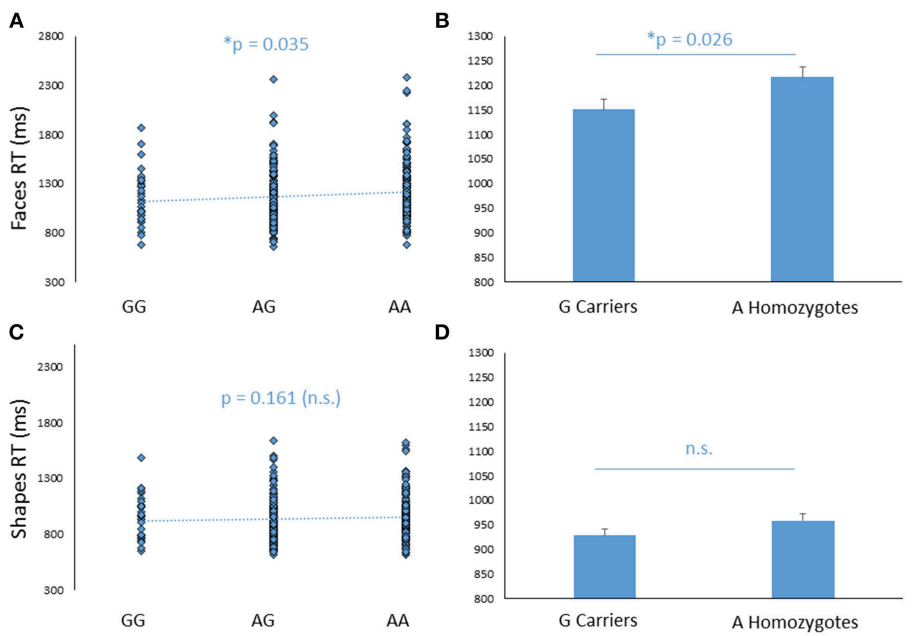

FIGURE 3 | Effects of *FREM3* rs7676614 genotype on reaction times in the perceptual face matching task. Increasing number of A alleles was associated with slower reaction times in the Faces **(A)**, but not the Shapes **(C)** condition. These results were confirmed in a G allele dominant model, where A homozygotes showed slower reaction times than G carriers in the Faces **(B)** but not the Shapes condition **(D)**. Error bars represent standard error of the mean. $^{*}p < 0.05$, $^{\#}p < 0.10$.

homozygotes: 0.96 ± 0.19 s; G carriers: 0.93 ± 0.19 s; $F_{(1, 363)} = 1.97$, $p = 0.161$; controlling for gender and age: $F_{(1, 361)} = 2.22$, $p = 0.137$; **Figures 3B,D**]. Notably, RTs were slower overall in the Faces, compared to the Shapes condition, possibly reflecting the relative perceptual complexity of the stimuli to be matched [Faces: 1.80 ± 0.28 s; Shapes: 0.94 ± 0.19 s $F_{(1, 361)} = 0.826$, $p < 0.0001$]. Thus, perhaps the lack of genotype effect on RT in the Shapes condition may reflect performance ceiling effects. There were no differences in accuracy between genotype groups ($p > 0.27$).

Trail Making Test

To further probe the effect of *FREM3* genotype on perceptual processing speed, we compared genotype groups on their performance on the TMT Parts A and B, administered outside the scanner. Despite A homozygotes' being nominally slower to complete Trails A, the difference in performance reached only trend levels [A homozygotes: 22.71 ± 6.60 s; G carriers: 21.64 ± 5.79 s, $F_{(1, 355)} = 3.11$, $p = 0.079$; **Figure 4A**]. Larger genotype differences emerged in Trails B performance, where A homozygotes were slower to complete the task by an average of 2.14 s [A homozygotes: 46.02 ± 12.73 s; G carriers: 43.89 ± 11.03 s, $F_{(1, 355)} = 3.69$, $p = 0.056$; **Figure 4B**]. No additive effect of number of A alleles was observed on either task ($p > 0.12$). Notably, there were no genotype effects on number of errors in either Trails A or Trails B ($p > 0.39$) and the differences in speed occurred in the absence of genotype differences in estimated IQ (**Table 1**). Furthermore, the effects remained trending when IQ was controlled for alongside gender and age (Trails A: $p = 0.089$; Trails B: $p = 0.076$).

There was no association between amygdala reactivity and processing speed, as reflected by either RT or TMT, either in the overall sample or for any specific genotype group ($p > 0.10$).

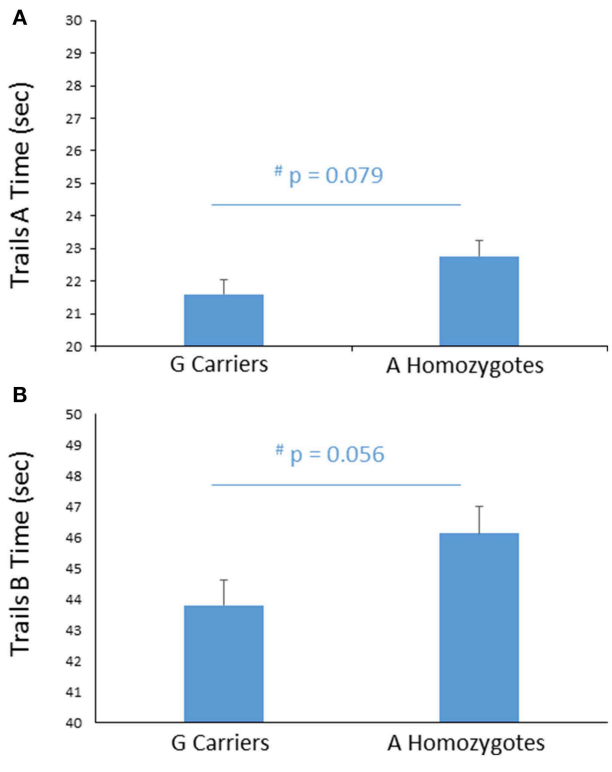

FIGURE 4 | Effects of *FREM3* rs7676614 genotype on TMT performance. Relative to G carriers, A allele homozygotes showed a trend toward slower performance in both Part A **(A)** and Part B **(B)** of the TMT. Error bars represent standard error of the mean. $^{*}p < 0.05$, $^{\#}p < 0.10$.

Postmortem Gene Expression

We next probed the effect of age and *FREM3* genotype on *FREM3* mRNA levels in human postmortem tissue. Age was associated with a robust decline in *FREM3* expression in both BA11 and BA47 following a near-linear trajectory (**Figures 5A,B**). In addition, our proxy SNP showed a trending effect, such that the major allele was associated with relatively reduced gene expression in both BA11 ($p = 0.062$; **Figure 5C**) and BA47 [**Figure 5D**, $p = 0.08$; $p = 0.066$ based on an adaptively-weighted (AW) meta-analysis (Li and Tseng, 2011) across both regions] in a pattern reminiscent of that seen in older age. Importantly, these results were adjusted for gender and estimated effects of chronological age at time of death.

Additional FREM3 Variants

To further explore the potential involvement of the *FREM3* gene in regulating depression-relevant neural and behavioral phenotypes, we identified rs1391187 as the SNP most strongly modulating *FREM3* expression across both BA11 ($p = 2.17 \times 10^{-5}$, **Figure 5E**) and BA47 ($p = 2.47 \times 10^{-6}$, **Figure 5F**, Supplementary Table 2) in our postmortem cohort, and tested its effects (via proxy SNP rs1909022, $R^2 = 1.00$, $D' = 1.00$), on neural activity and perceptual processing speed in our *in vivo* cohort. In striking similarly to rs7676614, the lower-expressing major allele at rs1391187/rs1909022 was also associated with reduced amygdala reactivity bilaterally (left amygdala: $b = -0.167$; $p = 0.001$; right amygdala: $b = -0.159$; $p = 0.002$; **Figures 6A,B**) as well as slower speed in the TMT Part B ($b = 0.114$; $p = 0.025$, **Figure 7A**). These effects remained significant when controlling for gender and age (left amygdala: $b = -0.170$; $p = 0.001$, right amygdala: $b = -0.162$; $p = 0.002$; Trails B, adjusted for IQ: $b = 0.111$; $p = 0.028$). Notably, there were no significant differences in gender composition or estimated IQ among rs1909022 genotype groups (**Table 2**). While there was a main effect of age ($p = 0.045$), such that heterozygotes were slightly but significantly older than GG homozygotes ($p = 0.024$, LSD-corrected), there was no linear association between number of minor/major alleles and age ($p = 0.486$).

There was no effect of rs1391187/rs1909022 genotype on RT in the Faces or Shapes matching condition or the TMT Part A ($p > 0.20$). Genotype groups did not differ on self-reported stress ($p > 0.30$) and stress did not interact with rs1391187/rs1909022 genotype to predict amygdala reactivity or processing speed ($p > 0.10$). Notably, rs1391187/rs1909022 and rs7676614 are not in high LD ($R^2 = 0.182$, $D' = 0.481$, 1000 Genomes) and did not interact with each other to predict any phenotype of interest ($p > 0.19$), suggesting their contributions to differences in *FREM3* expression and function may be independent.

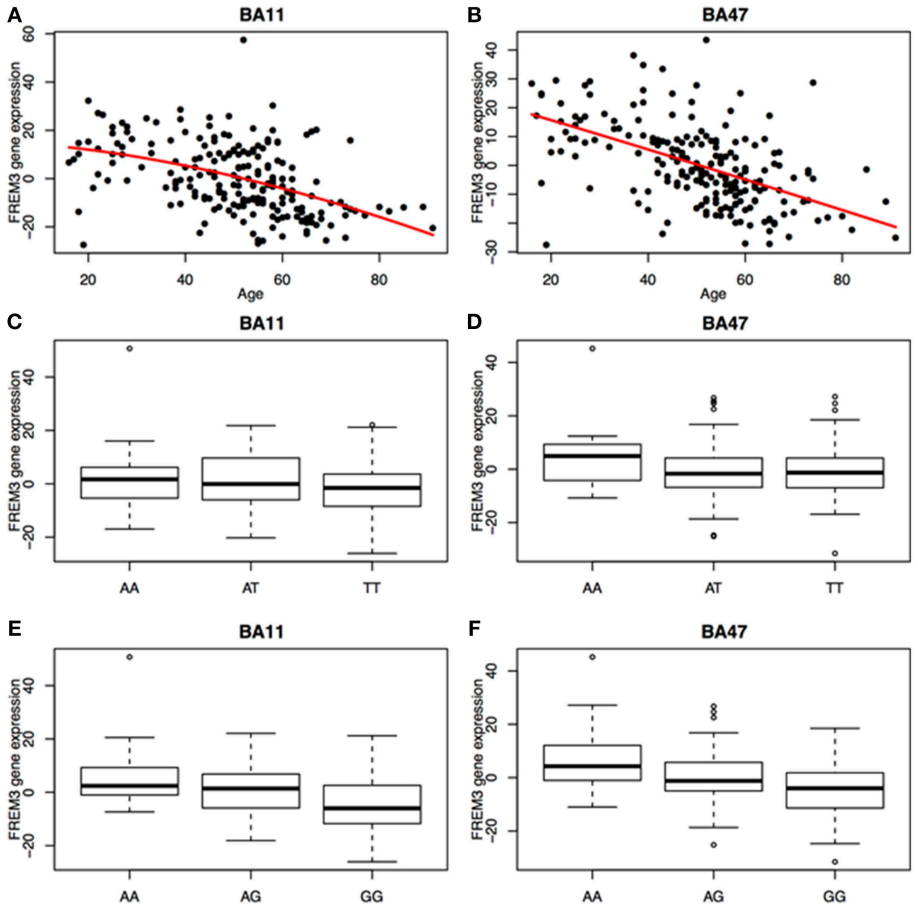

FIGURE 5 | Age was associated with a robust decrease in *FREM3* gene expression in both BA11 (A) and BA47 (B). In addition, the major T allele of rs6537170 (proxy for rs7676614), was associated with trend-level reductions in gene expression in BA11 **(C)** and BA47 **(D)**. Finally, the major G allele of rs1391187 was also associated with decreased gene expression in BA11 **(E)** and BA47 **(F)**. Each box in **(C–F)** represents the interquartile range of the data and the whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box.

An exploratory whole-brain analysis revealed no effect of *FREM3* rs1391187/rs1909022 genotype on activity in any other brain region. However, ROI analyses revealed that the major allele was associated with significantly decreased activity in BA47 (**Figures 6C,D**).

Link to Self-report Measures

Although there were no differences in measures of current mood and neuroticism between genotype groups as defined by either SNP ($p > 0.10$), increasing number of major alleles at rs1391187/rs1909022 was associated with lower extraversion, as assessed by the NEOE (no covariates: $b = -0.121$, $p = 0.021$, with covariates; $b = -0.124$, $p = 0.017$, **Figure 7B**). This effect was driven primarily by the Activity subscale (no covariates: $b = -0.143$, $p = 0.006$; with covariates: $b = -0.145$, $p = 0.005$). Furthermore, NEOE was positively correlated with BA47 activity in the left hemisphere, such that BA47 activity negatively mediated the association between number of rs1391187/rs1909022 major alleles and NEOE scores (**Figure 8A**). Finally, there was a positive correlation between amygdala reactivity and BA47, such that when amygdala and BA47 activity were entered sequentially into a mediation model, they were both found to be significant mediators of the link between rs1391187/rs1909022 genotype and NEOE (**Figure 8B**). Importantly, a model where amygdala reactivity was entered as a mediator more proximal to extraversion did not result in significant mediation ($b = 0.098$, $SE = 0.264$, 95% CI: –0.366, 0.704).

Discussion

In this study we show that the major A allele of the *FREM3* rs7676614 polymorphism is associated with blunted amygdala reactivity to socially salient stimuli, as well as reduced processing speed, as indexed by slower reaction time in a perceptual matching task and visuomotor performance in the Trails Making Test. These results are consistent with prior epidemiological and clinical studies associating the A allele with relatively heightened risk of depression (Muglia et al., 2010) as well as symptoms of psychomotor retardation (Shi et al., 2012). We

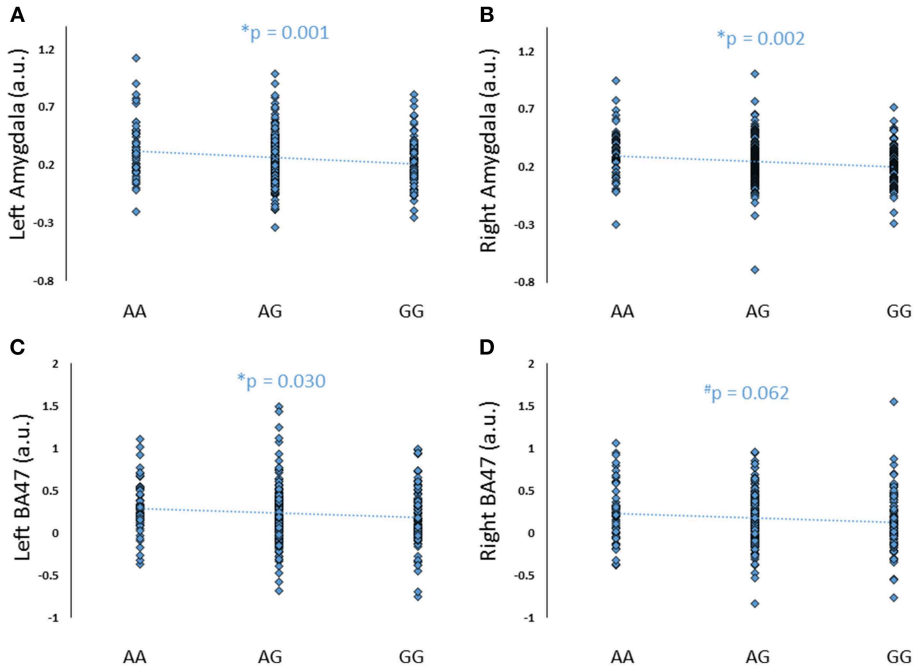

FIGURE 6 | Effects of *FREM3* rs1909022 genotype (proxy for rs1391187) on amygdala and BA47 activity (A–D). Increasing number of major (G) alleles was associated with reduced reactivity in both brain regions and hemispheres. $^{*}p < 0.05$, $^{\#}p < 0.10$, a.u. = arbitrary units.

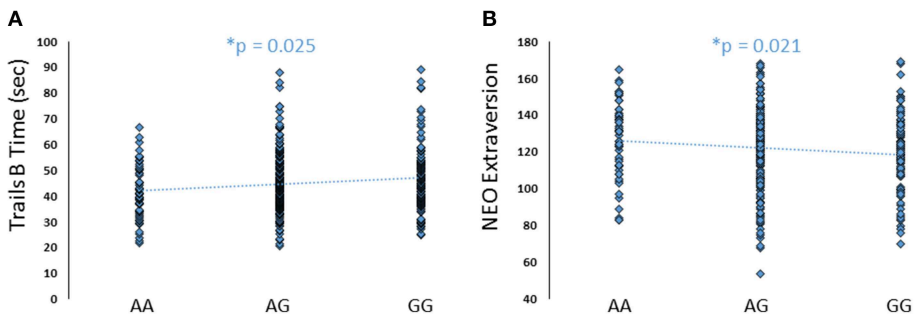

FIGURE 7 | Effects of *FREM3* rs1909022 genotype on TMT Part B performance and extraversion. Increasing number of major (G) alleles was associated with relatively slower TMT performance **(A)** and lower levels of extraversion **(B)**. $^{*}p < 0.05$.

also confirm and extend prior basic molecular work (Glorioso et al., 2011) by showing that *FREM3* expression in postmortem human prefrontal cortex (BA11 and BA47) tissue robustly decreases with age. Moreover, we show that the A allele of rs7676614 is associated with a trend toward reduced *FREM3* gene expression, shifting this molecular phenotype toward a pattern reminiscent of older age. Finally, we demonstrate convergent *in vivo* phenotypic effects of rs1391187, the SNP showing the strongest modulatory impact on *FREM3* expression in our postmortem cohort. Specifically, we show that the lower-expressing major allele of rs1391187 (assessed via proxy SNP rs1909022) is associated with relatively reduced amygdala reactivity, slower visuomotor performance in the TMT Part B, blunted activity in BA47, as well as reduced extraversion. Taken together, and in light of prior work demonstrating reduced amygdala reactivity and slower processing speeds in healthy older adults (Tessitore et al., 2005), our results suggest that the major alleles at both rs7676614 and rs1391187/rs1909022 may increase depression risk by precipitating the premature activation of biological pathways implicated in the normal molecular aging of the brain (Sibille, 2013). Conversely, the minor alleles at these loci may be associated with relative resilience to depression via a comparative slowing down of normal age-related processes.

The conceptual link between depression and accelerated aging (Wolkowitz et al., 2011; Sibille, 2013) may seem at odds with epidemiological data suggesting MDD is diagnosed less frequently in older, relative to young adults (Hasin et al., 2005). This seeming inconsistency may be due to the fact that late-life depression has a unique symptom profile, only partially

TABLE 2 | Summary of demographic characteristics and estimated IQ represented as a function of *FREM3* rs1909022 genotype.

	FREM3 rs1909022			*p*	*p* (additive)
	GG	AG	AA		
n	111	197	57	‘	‘
Male/Female	49/62	100/97	24/33	0.367	‘
Age (mean ± SD)	19.58 ± 1.18	19.90 ± 1.24	19.60 ± 1.24	**0.045**	0.486
Estimated IQ	123.12 ± 6.96	124 ± 7.51	123.37 ± 7.57	0.575	0.651
CTQ	31.67 ± 7.34	31.11 ± 7.49	32.18 ± 7.48	0.591	0.857
LESS	4.16 ± 2.97	4.23 ± 3.13	4.26 ± 2.43	0.974	0.821
NEO-N	84.19 ± 23.90	81.51 ± 21.67	80.40 ± 22.66	0.494	0.253
NEO-E	119.11 ± 19.55	121.67 ± 21.88	127.35 ± 20.23	0.055	**0.021**

The table includes p-values from both a mean comparison (ANOVA) and an additive (regression) model. Significant values are highlighted in bold font.

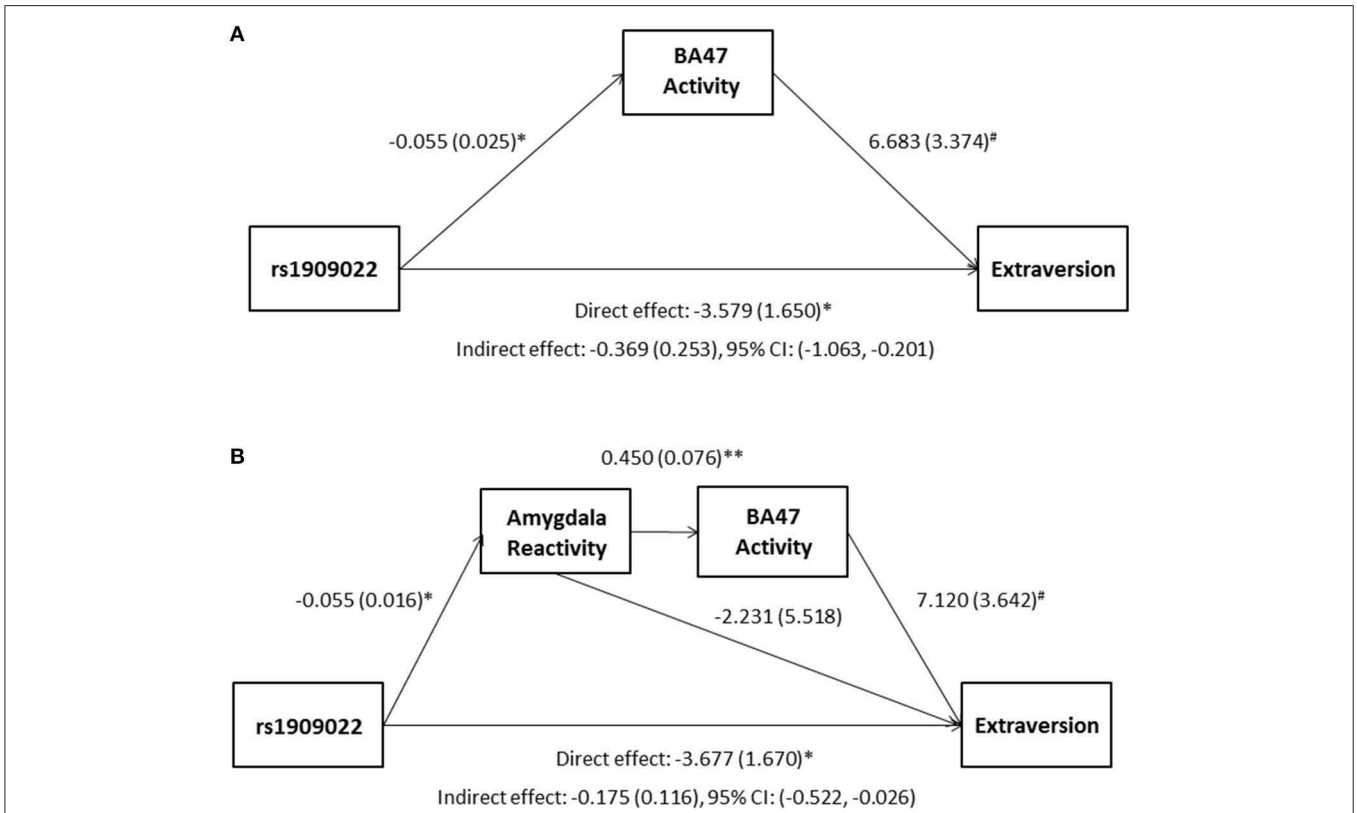

FIGURE 8 | Results from mediation models wherein activity in BA47 mediated the association between rs1909022 genotype and extraversion scores by itself (A) or in conjunction with amygdala reactivity (B). Raw regression coefficients are presented for each path, along with standard errors in parentheses. Bootstrapped 95% confidence intervals are presented for the indirect (mediation) effects. $^{**}p < 0.005$, $^{*}p < 0.05$, $\#p < 0.10$. CI, Confidence interval.

overlapping with that seen in young and middle-aged adults. For example, depressed older adults are less likely to report affective symptoms, which are heavily weighted in DSM criteria for MDD (Fiske et al., 2009). In fact, older adults generally report greater well-being (Carstensen et al., 2011). However, they are more likely to experience depression-related cognitive and somatic symptoms, including psychomotor retardation, as well as loss of interest in normal daily activities (Fiske et al., 2009). These symptoms may in turn contribute to subsyndromal depression, which may cause significant impairment in the quality of life of the elderly, without necessarily meeting DSM diagnostic criteria. Because depressive symptoms in the elderly are frequently distinct from those experienced by younger individuals and tend to be misattributed or overattributed to physical illness or bereavement, depression or subsyndromal depressive symptoms which impair quality of life in the elderly may be underdiagnosed and untreated (VanItallie, 2005). Moreover, early- and late-life onset depression likely represent distinct biological entities with

different underlying pathophysiologies. Our findings conceivably tap into the pathophysiology of late-life onset depression, risk for or resilience to which may, however, be detectable at a younger age (Douillard-Guilloux et al., 2013).

Abnormally increased (Whalen et al., 2002; Victor et al., 2010; Yang et al., 2010) or decreased (Thomas et al., 2001; Lawrence et al., 2004; Wolfensberger et al., 2008) amygdala reactivity to threat or other emotionally salient stimuli have both been implicated in depression or risk thereof. The mixed nature of these findings may reflect distinct depression subtypes or pathways of risk. Heightened amygdala reactivity may be associated with increased sensitivity to environmental events and thus particularly predispose to depression in the context of stress (Caspi et al., 2010). In contrast, a relative blunting of amygdala response may reflect a reduced reactivity to environmental salience, which can be a risk factor for depression in its own right (Rottenberg et al., 2005; Bylsma et al., 2008). Our results suggest that the low-expressing major alleles at both rs7676614 and rs1391187/rs1909022 may confer risk for a subtype of MDD via the latter pathway. This notion is further strengthened by the fact that we observed no significant interaction between genotype and self-reported stress in predicting amygdala reactivity. Finally, this conceptualization is also consistent with a recent twin study which demonstrated that non-overlapping contributions of genetic and environmental factors to depression risk are associated with amygdala hypo- and hyper-reactivity, respectively (Wolfensberger et al., 2008). Taken together, these data suggest that *FREM3* functional variation may uniquely contribute to risk for a subtype of depression that is not precipitated by stress, but is rather a more proximal result of genetic influences. It should, however, be noted that effects of early life stress may emerge in a sample reporting higher and/or more variable levels of childhood trauma.

Importantly, *FREM3* is among the genes whose expression in the amygdala and ACC has previously been shown to decrease as a function of age (Glorioso et al., 2011). However, this prior work has used a cohort which may have been underpowered to detect changes in gene expression occurring across the entire adult lifespan. Here, using a cohort spanning the entire adult age range (16–96 years old), we extend these prior findings by reporting a robust and highly significant age-associate decrease in *FREM3* expression in Brodmann areas 11 and 47—regions broadly overlapping with the orbitofrontal cortex (OFC) as well as the ventrolateral prefrontal cortex (vlPFC), both of which have previously been implicated in depression pathophysiology and risk (Rogers et al., 2004; Stuhrmann et al., 2011).

Extensive prior work has shown that a shift toward a premature activation of age-related molecular pathways may increase risk for depression, among other neuropsychiatric disorders (Douillard-Guilloux et al., 2013; Sibille, 2013). In the current study, we report that the rs7676614 A allele, as indexed by the major allele of the proxy SNP rs6537170, is associated with a trend toward decreased *FREM3* expression in postmortem human brain tissue, independent of chronological age. This finding suggests that the A allele may predispose to depression via shifting *FREM3* expression to a pattern reminiscent of that seen in older age. Conversely, the minor G allele may be associated with relative resilience to the activation of these pathways and effectively contribute to a protective "slowing down" of normal age-related changes. Consistent with this conceptualization, one prior study has demonstrated a link between normal aging and the same *in vivo* phenotypic patterns we associated with the A allele (Tessitore et al., 2005). Specifically, using a BOLD fMRI paradigm similar to the one employed here, this study showed a relative reduction in amygdala reactivity and a concomitant slowing of face- but not shape-matching reaction times in older, relative to young, adults (Tessitore et al., 2005). Importantly, and in line with the current findings, this occurred in the absence of any differences in face or shape matching accuracy. Phenotypic convergence between older adults and carriers of the rs7676614 A allele is particularly remarkable in light of the fact that our sample consisted exclusively of young adults between the ages of 18 and 22.

The finding of slower reaction times in the face-matching condition in individuals with the rs7676614 A allele is further consistent with prior associations of the same allele and symptoms of psychomotor retardation (Shi et al., 2012). Furthermore, we found convergent effects of genotype on performance in the TMT, which is another index of psychomotor function. Importantly, we found a stronger genotype effect on performance in Trails B than in Trails A. Part A of the TMT is generally interpreted as a purer measure of motor function, while Part B taps into more complex visuomotor skills and task switching (Bowie and Harvey, 2006). The stronger genetic effect we observed in Part B, relative to Part A, suggests that rs7676614 may modulate higher-order cognitive processing of perceptual stimuli, rather than motor function alone. This notion is also consistent with the specificity of the rs7676614 effects on reaction times to the more perceptually and cognitively challenging face-matching condition, rather than the shape-matching condition of our amygdala reactivity paradigm.

While we found that the major allele of rs1391187/rs1909022, which is even more strongly linked to reduced *FREM3* expression, is similarly associated with reduced amygdala reactivity and slower TMT Part B performance, we found no effect of rs1391187/rs1909022 on RT in the Face or Shape matching condition or TMT Part A. It is possible that this SNP, due to its stronger impact on *FREM3*, may even more selectively impact complex, relative to simple, visuomotor tasks. The partial discrepancy in phenotypic association between rs7676614 and rs1391187/rs1909022 may also be attributed to differential LD patterns with additional variants, perhaps impacting the expression of neighboring genes.

Despite the effect of genotype on both neural reactivity and processing speed, we did not observe an association between TMT speed and RT and activity in either amygdala or BA11/47. Even though we investigated the effects of our SNPs of interest on gene expression only within regions of the OFC and vlPFC, *FREM3* is expressed in multiple brain regions (Hawrylycz et al., 2012). Thus, it is possible that the genetic effects on processing speed are primarily mediated through brain regions or systems not directly activated by our fMRI paradigm.

Importantly, we show that rs1909022, the SNP with a more robust effect on postmortem *FREM3* expression, is not

only associated with differential amygdala response, but also modulates activity in regions of the OFC and vlPFC overlapping BA47. Specifically, we demonstrate that the low-expressing major allele is associated with blunted response to salient facial affect cues in BA47. Prior work has shown decreased OFC activity to negative (sad and angry) facial expressions in MDD (Lee et al., 2008), which may at least partially account for the deficits in emotion recognition and reactivity observed in currently depressed individuals (e.g., Persad and Polivy, 1993; Asthana et al., 1998). Other studies demonstrate OFC hyporeactivity may persist after disorder remission, but is remediated by acute administration of the antidepressant citalopram (Anderson et al., 2011). Additional research suggests that BA47 may be involved in selectively incorporating emotion into decision making (Beer et al., 2006). Specifically, activity in this region has been shown to increase when incorporating relevant negative emotional information into behavior, as well as when successfully inhibiting irrelevant negative information (Beer et al., 2006). Thus, hypoactivity in the same region may be associated with maladaptive use of emotion to guide behavior and constitute a risk factor for the development of depression. Furthermore, additional prior MRI research indicates that the structural and functional properties of the OFC may be particularly vulnerable to age-related decline, relative to other parts of the PFC (Lamar and Resnick, 2004; Resnick et al., 2007).

In addition to the effects of rs1909022 genotype on BA47 activity, we also observed a positive correlation between BA47 activity and extraversion. Prior work has demonstrated that higher extraversion may be protective against MDD, as well as psychopathology more broadly (Campbell-Sills et al., 2006). This, together with the fact that we associated the more common alleles at both loci with a relative blunting of neural reactivity, as well as slower processing speed, lends support to the conceptualization that the minor alleles may in fact be conferring relative resilience against depression- and age-related processes above and beyond what may be a normal "baseline" phenotype associated with the major alleles.

We further demonstrated that there was a positive correlation between amygdala response and BA47 activity, and, when taken together, these neural phenotypes both mediated the association between rs1909022 genotype and extraversion. The convergent effects of rs1909022 on both neural phenotypes, as well as their joint mediation effect, suggest that functional variation impacting *FREM3* expression likely modulates activity in multiple brain regions. Future work applying functional connectivity analytic approaches to task-based or resting state brain activity on the network level is likely to shed additional light on the full scope of these effects.

It should be noted that despite the fact that we observed a main effect of task, as well as an effect of rs1391187/rs1909022 genotype on gene expression, in both BA47 and BA11, we showed an effect of genotype on activity in BA47, but not BA11. Given the more ventral position of BA11, this region is more prone to signal dropout due to its proximity to tissue boundaries. Thus, it is possible that the most strongly activated and functionally relevant regions within BA11 were not adequately covered by our BOLD fMRI sequence. Future studies using imaging sequences optimized for coverage in the ventral OFC are likely to shed more light on the relative specificity of these and similar findings to lateral vs. more ventral regions of the OFC.

Critically, we found no direct link between genotype and current depressive or anxious symptomatology. However, it should be noted that our *in vivo* sample consisted of high-functioning young adults attending a competitive university. Thus, it is possible they are a particularly resilient population with relatively limited variability in depressive and anxious symptoms. Similarly, our postmortem sample contained individuals free of neuropsychiatric illness. In light of these sample characteristic, it might be reasonable to suggest that the genetic effects we observe on amygdala reactivity, perceptual processing speed, as well as gene expression, may reflect a latent vulnerability, which may only lead to disorder in the context of additional risk factors, including, but not limited to, increasing chronological age. Importantly, we did observe a link between the major allele of rs1391187/rs1909022 and the personality trait extraversion, partially mediated via differences in BA47 activity. Given prior links between extraversion and resilience to psychopathology independent of risk conferred by other personality traits (Campbell-Sills et al., 2006), our results may reflect a resilience phenotype associated with the high-expressing, rather than a vulnerability phenotype associated with the low-expressing *FREM3* alleles. Future studies utilizing prospective longitudinal designs, especially in cohorts enriched in elderly subjects and/or for MDD-related pathology, will be necessary to ascertain the validity of this notion.

The molecular mechanisms underlying the associations reported here have yet to be elucidated. The human *FREM3* gene codes for an extracellular matrix protein belonging to the same family as FRAS1, FREM2, and QBRICK/FREM1, which have been implicated in Fraser syndrome—a disorder characterized by severe congenital malformations in the development of the nose, ears, and throat, as well as mental retardation (van Haelst et al., 2007). However, no evidence has directly linked *FREM3* to Fraser syndrome and additional research in mouse models suggests the murine Frem3 gene shows distribution patterns distinct from those of Frem1 and Frem2 (Kiyozumi et al., 2007). As an extracellular matrix protein expressed in the brain, FREM3 is likely implicated in cell–cell interactions and maintaining the structural and functional integrity of nervous tissue. Thus, it is likely to be involved in neural development and function. Importantly, the extracellular matrix and its associated proteins have been shown to play an important role in brain aging (Morawski et al., 2014). Consistent with this, additional extracellular matrix proteins such as reelin also show strong, albeit less significant, age-related trajectories (Erraji-Benchekroun et al., 2005; Glorioso et al., 2011). Future studies directly manipulating the Frem3 gene in animal models would be needed to elucidate the role of this gene in normal and pathological brain function.

While both rs7676614 and rs1391187 were found to modulate *FREM3* gene expression in postmortem brain tissue, the precise molecular mechanisms via which these polymorphisms may exert their effects are unclear. Both rs7676614 and rs1391187, as well as their respective proxy SNPs rs6537170 and rs1909022, are

intronic; thus, they may be involved in regulating alternative gene splicing. However, follow-up molecular genetic studies would be necessary to confirm this conjecture.

This study is not without its limitations. First, in order to ensure adequate statistical power and consistency with prior genetic association studies, we focused our analyses on non-Hispanic Caucasian individuals as they were the largest single ethnic group represented across samples. However, future studies should incorporate other ethnicities in order to more fully characterize the role of *FREM3* on depression risk. In addition, while we focused our *in vivo* analyses on the amygdala, we were unable to assess SNP effects on gene expression in that brain region. Prior work has demonstrated age-related decreases in *FREM3* expression in the amygdala and ACC (Glorioso et al., 2011), so it may be reasonable to assume that the cis-eQTL effect observed here also occur in the amygdala, although this will need to be tested directly. Indeed, drawing on prior work suggesting a general convergence of depression-associated molecular pathway dysregulation across diverse brain regions such as the ACC (Tripp et al., 2011), dorsolateral prefrontal cortex (dlPFC) (Sibille et al., 2011), and the amygdala (Sibille et al., 2009; Guilloux et al., 2012), we believe *FREM3* genetic variation would similarly impact expression patterns throughout the majority of cortical structures implicated in depression. Detailed molecular studies utilizing samples collected from different brain regions, ideally within the same individuals, would be necessary to test this conjecture.

In addition, while significant, the phenotypic differences we observed among genotype groups in our *in vivo* cohort were relatively small in size. This may be due to the fact that our sample consisted of high-functioning young adults with higher than average IQ, who were mostly free of current or past depressive symptomatology (Supplementary Table 1). Nonetheless, the fact that significant, albeit small, genetic differences emerged across multiple depression-relevant phenotypes even in this young and highly resilient sample may suggest that a potentially greater contribution of *FREM3* to depression risk could be unmasked in older and/or longitudinal samples, as well as those enriched for psychopathology. Our results should be interpreted with caution until confirmed by future research in such samples.

Last but not least, we focused on two polymorphic loci within a single gene, while our phenotypes of interest are known to be highly polygenic in nature. In this study, however, we did not set out to exhaustively explain phenotypic variability, but, rather, to illustrate how convergent results from multi-modal mechanistic follow-up studies and partial replications of suggestive GWAS hits may serve to improve biological knowledge of depression risk pathways. This knowledge can in turn be used to inform and guide future GWAS through phenotypic refinement or the enhancement of emergent variant prioritization techniques (e.g., Gagliano et al., 2014). Such synergy between large-scale genetic association studies and more targeted mechanistic approaches may not only offer better opportunities for the discovery of novel risk variants, but also promote an increasingly detailed delineation of biological pathways of risk, which may in turn open novel avenues for early individualized treatment and, possibly, prevention.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpsyg.2015.01377

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Hypothesis-driven research for G × E interactions: the relationship between oxytocin, parental divorce during adolescence, and depression in young adulthood

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Research in molecular genetics has generally focused on genome-wide association studies (GWAS) and exploratory candidate gene and candidate gene–environment (G × E) studies. In this article it is proposed that hypothesis-driven and biologically informed research provides a complementary approach to GWAS to advance pressing research questions about G × E relations that are of public health relevance. Prior research studies and developmental and evolutionary theory were used to guide hypothesis testing of G × E relationships in this study. The study investigated whether the oxytocin polymorphism, rs53576, moderated the relationship between parental divorce during adolescence and depression symptoms in young adulthood. Oxytocin is a neuropeptide that has been related to the regulation of complex social cognition and behaviors such as empathy, attachment, and nurturance. We hypothesized that the GG polymorphism would be associated with more depressive symptoms following parental divorce, and that this effect would be stronger in females than males. The sample consisted of 340 individuals who participated in a longitudinal study with data used both from adolescence and young adulthood. Findings using prospective follow-up and autoregressive change models supported the hypothesized relationships. Young adult females who had experienced parental divorce during adolescence and had the GG oxytocin genotype reported almost twice as many depressive symptoms relative to young adult females who also experienced parental divorce during adolescence but had the AA or AG genotype. This pattern was not indicated among males. Findings were discussed with regard to how molecular genetic factors in combination with environmental stressors, such parental divorce, framed within a developmental framework may facilitate the future study of G × E relationships in the parental divorce-child adjustment literature and contribute to a prevention science perspective.

Keywords: parental divorce, adolescence, depression, oxytocin, young adulthood

Introduction

An extensive literature on parental divorce has indicated that youth from divorced families more frequently report greater internalizing (e.g., depression, distress, and pain) and externalizing (e.g., delinquency, oppositional behaviors) problems, lower academic performance, and more interpersonal difficulties than children or adolescents from intact families (Cherlin et al., 1998; Booth and Amato, 2001; Lansford, 2009). For example, using long-term prospective data, Hetherington and Kelly (2002) reported that 25% of offspring from divorced families had long-term psychological and social problems relative to only 10% of offspring from families where parents remained together. Similarly, prospective findings by Cherlin et al. (1998) indicated that mental health difficulties among children of divorce relative to children of intact families increased across time when offspring were in their 20 and 30 s. Moderators and mediators of offspring outcomes associated with parental divorce have included factors such as parental conflict preceding the divorce, children's level of adjustment preceding divorce, and post-divorce parent–child relations (Booth and Amato, 2001; Lansford, 2009). Studies utilizing behavior genetic research designs have supported both genetic and environmental influences on child adjustment following parental divorce (McGue and Lykken, 1992; O'Connor et al., 2000). However, to our knowledge, no study in this area of research has examined how molecular genetic factors may moderate the prospective relationships between parental divorce that occurred for offspring during adolescence, and subsequent depressive symptoms of these offspring during young adulthood.

Research suggests that polymorphisms of the oxytocin receptor gene are involved in the regulation of complex social cognition and behaviors, including empathy, attachment, and nurturance (Bakermans-Kranenburg and van IJzendoorn, 2008; Buchheim et al., 2009; Meyer-Lindenberg et al., 2011). The potential moderating role that a polymorphisms of oxytocin (OXTR), rs53576 (GG), may have in relation to a significant adolescent stressful life event (parental divorce) in terms of depression in adolescence and young adulthood has not been investigated and is the focus of this study.

Beginning in early adolescence, sex differences emerge with regard to depressive symptoms, with girls manifesting significantly higher levels than boys; these sex differences are exacerbated during middle adolescence and are maintained across time with adult women having about twice the rate of major depressive disorders as men (Hankin et al., 1998; Essau et al., 2010). A range of different hypotheses have been forwarded to account for the emergence of sex differences in depression during adolescence (Hankin et al., 1998; Nolen-Hoeksema, 2001; Hyde et al., 2008; Essau et al., 2010). For instance, the onset and development of puberty and associated perceptions of self-esteem and body image differ for boys and girls, with both increased size and masculine characteristics viewed positively by boys but increased size and weight often viewed negatively by girls. These changes associated with pubertal development may contribute to increased discrepancies in depressive symptoms between boys and girls.

Another hypothesis suggests that the adoption and incorporation of gender roles during adolescence, referred to as "gender role intensification" (Hill and Lynch, 1983), may differentially impact boys and girls. Gender role intensification describes male development during this period as being more outwardly focused on autonomy, competitiveness, and self-confidence, whereas female development is more inwardly focused on emotional expressivity, warmth, and care of and support for others. Thus, a major disruptive event such as parental divorce may differentially impact age-appropriate developmental role performance for boys and girls, thereby contributing to higher levels of depression among females. Findings by Mustonen et al. (2011) indicated that parental divorce during adolescence was associated prospectively with poorer intimate relationship quality in adulthood (16-year follow-up) among daughters than sons.

Yet another perspective on gender differences in depression is provided by an evolutionary life history framework for psychopathology (Del Giudice, 2014). Prior research has suggested that men are more susceptible to depression due to status loss, whereas women are more susceptible due to reduced social support (Kendler et al., 2005; La Greca et al., 2009). Building upon these findings, Del Giudice (2014) has incorporated stress-related components into his model of depression, in addition to affective reactivity, and has emphasized the need to investigate both affective reactivity and stress reactivity in relation to slow or fast life history strategies. Furthermore, it is proposed that across development men develop more unemotional responses characterized by a more hypo-responsive stress response system whereas females may develop more hyper-responsive stress systems. The occurrence of a major stressor such as parental divorce makes the environment less stable and more chaotic, whereby social support and stable intimate relationships can decrease rapidly. Because females have more hyper-responsive stress response systems than males, and are differentially affected by disrupted social environmental factors (e.g., social support from parents and family members), the expectation would be that females would respond more adversely to parental divorce than males through the expression of more depressive symptoms.

There is increasing evidence that sub-threshold levels of depressive symptoms (i.e., expressing some depressive symptoms but not a sufficient number to meet clinical diagnostic criteria) in adolescence are clinically significant in predicting subsequent mental health problems in young adulthood (Lewinsohn et al., 2000; Cuijpers and Smit, 2004). For example, findings by Gotlib et al. (1995) indicated that adolescents with high scores on the Center for Epidemiologic Studies Depression Scale (CES-D; Radloff, 1977) who did not meet formal clinical diagnostic criteria for a depressive disorder did not differ from adolescents meeting these criteria on several measures of psychopathology and psychosocial dysfunction. Furthermore, several two-wave prospective studies indicated that elevated, but subclinical depressive symptoms in adolescence were a risk factor for the development of adult depressed mood (Kandel and Davies, 1986) and adult depressive disorder (Rao et al., 1999; Cuijpers and Smit, 2004). The implication of these

findings is that high levels of depressive symptoms among adolescents, as reported on self-report measures such as the CES-D, pose a serious public health problem among adolescents in transition to young adulthood in that they may predict aggravated mental health problems and disorders through the lifespan.

Oxytocin is a neuropeptide that impacts a range of response systems including some related to reproduction (uterine contraction during childbirth) and early childhood care (e.g., milk ejection during breastfeeding) and others associated with a range of social cognitive and social affiliative behaviors (Bakermans-Kranenburg and van IJzendoorn, 2008; Costa et al., 2009; Meyer-Lindenberg et al., 2011). The rs53576 SNP is located on chromosome 3p25 and has unknown biological functionality in the third intron of the larger oxytocin gene (OXTR). Research findings for rs53576 and social affiliative behaviors have been consistent in demonstrating significant statistical associations, but the identified risk alleles of those associations have not always been consistent. For example, Rodrigues et al. (2009) reported that A allele carriers had lower empathy and lower positive affect, and manifested greater physiological stress reactivity. Similarly, Bakermans-Kranenburg and van IJzendoorn (2008) reported that carriers of the AA/AG genotypes demonstrated lower levels of maternal responsiveness to toddlers. By contrast, Costa et al. (2009) found that GG allele carriers had higher levels of separation anxiety in adulthood, including a higher need for social approval and lower confidence with respect to having a secure attachment. Norman et al. (2012) also provided evidence that GG carriers manifested higher levels of sympathetic reactivity to psychological stress.

Although these sets of findings are in conflict, there is emerging evidence that inconsistencies may be attributable to genotype × environment interactions. Chen et al. (2011) used a laboratory design to investigate the possibility that rs53576 might interact with stress-protective effects of social support. They found that social support prior to a laboratory-based stressor was associated with lower cortisol and subjective stress responses for G allele carriers, but not for AA carriers. Hence, there was a genotype by stress (environment) interaction effect. Similarly, Sturge-Apple et al. (2012) reported a statistically significant interaction for oxytocin × interparental conflict on maternal sensitivity with toddlers. Across low and high levels of interparental conflict, AA/AG carriers did not differ in maternal sensitivity to their toddlers; however, GG carriers demonstrated plasticity across levels of interparental conflict with low levels of conflict associated with high sensitivity and then sensitivity decreasing significantly as interparental conflict increased. Bradley et al. (2011) reported an interaction between childhood maltreatment and the rs53576 SNP in that GG genotype carriers were at increased risk for emotional dysregulation and a disorganized attachment style relative to AA and AG carriers. These studies suggest that GG carriers may be most sensitive to positive and negative environmental influences. Collectively, these findings suggest the potential benefits of considering gene × environment interactions when examining the role of oxytocin and stress on social affiliative behaviors. Furthermore, some findings suggest that sex may further moderate the strength of associations between oxytocin, stress, and social affiliative behaviors (Tost et al., 2010).

In this study, we used literature on sex differences in depression and differential responses to stressors, as well as literature on sex role socialization (e.g., gender role intensification) and evolutionary theory, as a basis to examine hypotheses about sex differences in the conditional (G × E interactional) relationship between parental divorce occurring during adolescence and the oxytocin polymorphism rs53576. Specifically, we hypothesized that divorce occurring during adolescence would interact with the rs53576 polymorphism to prospectively predict depressive symptoms among young adult females but not young adult males. To the extent that parental divorce during adolescence contributes to a greater disruption of the inwardly focused gender role developmental tasks (e.g., emotional expressivity, warmth, and care of and support for others) of females relative to the outward-focused male tasks, and reduces the quality of the social environment that is evolutionarily more important to females, we would expect greater increases in depressive symptoms in females. Additionally, the GG oxytocin genotype should make females even more susceptible to the negative effects of parental divorce. Thus, females with the GG genotype who also had experienced divorce during adolescence would be hypothesized to have significantly higher depression scores in young adulthood than females not homozygous for GG and all males.

Materials and Methods

Participants

The data used in this report were collected as part of a larger, multi-wave panel design study focused on risk and protective factors and adolescent and young adult substance use and mental health. We refer to the study by the acronym LAT, which stands for Lives across Time: A Longitudinal Study of Adolescent and Adult Development (for details, see Windle et al., 2005). Initially, data were collected from adolescents in their high school setting and the overall student participation rate was 76%. The average age of the respondents at the first occasion of measurement was 15.54 years ($SD = 0.66$) and 98% were white. Sample retention across the first four waves of measurement was uniformly high, in excess of 90%.

In this study we used data from 11th and 12th grade participants in all four adolescent waves (with 6-month intervals between assessments) and their follow-up young adult wave (Wave 5) approximately 6-years later when the average age of the young adults was 23.5 years. In-school surveys were completed during adolescence and individual interviews were conducted in young adulthood. For the current study, supplemental funding was provided by NIAAA to collect and analyze DNA for a subsample of the LAT (there were insufficient funds and an abbreviated time window to collect data from all participants). The subsample selected consisted of 340 participants with priority of selection given to subjects who participated during both adolescence and young adulthood to maximize the prospects of testing developmental gene–environment (G × E)

prospective relationships. The 340 individuals who participated did not differ significantly from those who did not participate on key variables (e.g., family income, adolescent alcohol use, adolescent depression, and grade-point average).

Procedure

During the adolescent phase, subsequent to receiving informed consent both from a parent and the target adolescent, a trained survey research team administered the survey to adolescents in large groups (e.g., 40–50 students) in their high school setting at each wave. The survey took about 45–50 min to complete and students received $10 for their participation. The study was approved by the University at Buffalo IRB and confidentiality was further assured with a U.S. Department of Health and Human Services Certificate of Confidentiality. The young adulthood interview at Wave 5 was conducted via one-on-one interviews either in the participants' homes or at the host institute of the investigators. Adults were paid $40 to complete an interview that lasted approximately 2 h. Computer-assisted personal interviews were used to collect the survey data.

Saliva samples were provided from a subset of the sample via a mail data collection protocol using Oragene DNA kits (Genetek; Calgary, AB, Canada). Participants were instructed to rinse their mouths with tap water and then deposit 4 ml of saliva in the Oragene sample vial. The vial was sealed, inverted, and shipped via courier first to the location of the study's primary investigator and then to the Georgia Genomics Facility (GGF) at the University of Georgia (http://dna.uga.edu) where DNA was extracted from saliva samples according to the manufacturer's specifications.

Measures

Sociodemographic Variables

In their individual interviews and the completion of mail surveys (during the adolescent phase of the study), participating parents were asked about their age, number of years of education completed, family income, and other demographics (e.g., marital and occupational status). Family income (during the target's adolescence) and highest level of education by either parent were used as covariates in data analyses.

Parental Divorce

Parental divorce or separation was assessed by an item within a list of 31 undesirable life events that was constructed by modifying the brief (24-item) form of the Adolescent Life Change Event Scale (ALCES; Forman et al., 1983) as detailed by Windle (1987). At each of the four waves of measurement that occurred during adolescence, adolescents were requested to report whether each of the life events occurred during the previous 6 months. The event was worded as "parental divorce or separation" with a Yes/No response format. Parental divorce was coded as a dichotomous variable indicating whether parental divorce or separation was endorsed at any of the four occasions. For this sample, 8.8% of the adolescents reported parental divorce across the 2-year interval. Parental divorce during adolescence as reported by adolescents was used in this study because of the proposed (adolescent) developmental significance in reference to the proposed hypotheses. The possible impact of parental divorce on child outcomes may vary contingent on age, sex, and a host of other variables that were beyond the scope of this study.

Depressive Symptoms

At Waves 4 and 5, depressive symptoms were assessed using the CES-D (Radloff, 1977). The CES-D consists of 20 self-report items and provides a unitary measure of current depressive symptoms, with an emphasis on the affective component, depressed mood. Participants were asked to indicate how many days during the past week they had experienced the emotions or behaviors indicated in each of the items. The response options for these items ranged from "0 = *Rarely or none of the time*" to "3 = *Most or all of the time*." The internal consistency estimate for the CES-D in this sample was 0.90 at Wave 4 and 0.91 at Wave 5.

Oxytocin (OXTR)

DNA was extracted from saliva samples according to the manufacturer's specifications at the Georgia Genomics Facility (GGF) at the University of Georgia (http://dna.uga.edu). Genotyping was conducted by a technician blind to other data from the research project. High throughput genotyping was completed for single nucleotide polymorphisms (SNPs) on BeadXpress using the GoldenGate Genotyping Assay for VeraCode from Illumina. A customized set of SNPs was provided to Illumina by the investigator and Illumina provided the final oligonucleotides sequences to be used in the GoldenGate assay at the GGF. Quality control data procedures for the SNPs included a genotype call rate of 98% and subject filtering per SNP call rates equal-to-or-greater-than 95%. Exclusion of SNPs also occurred if the minor allele frequency rate (MAF) was less than 1%, there was significant departure from Hardy–Weinberg equilibrium at $p < 10^{-4}$, or outliers occurred (using a criterion of more than 5 SD). The OXTR rs53576 polymorphism was assessed as part of the SNP panel and met the criteria described above. The genotype distribution of OXTR for AA was 10.0% ($n = 34$), AG 41.2% ($n = 140$), and GG 48.8% ($n = 166$). Consistent with standard scoring for this SNP (e.g., Sturge-Apple et al., 2012), AA and AG genotypes were combined and compared with the GG group.

Data Analyses

Preliminary analyses examined frequency distributions of variables to identify outliers and influential data points, as well as non-normal distributions. For example, with respect to the latter, skewness for the depression scale at Wave 4 was –0.35 and at Wave 5 was 1.29. These values do not deviate largely from a symmetric distribution and the use of transformed variables for the Wave 5 depression score did not alter substantive findings from the use of raw scores. Also, no outliers or influential data points were observed for depression for children from divorced and non-divorced families. A linear regression model was used to specify two different prospective models to predict Wave 5, young adult depression. The first specified model included Wave 5 depression as the dependent variable and the predictor variables consisted of the two covariates of parental education and family income, the three "main effects" of Sex, OXTR (rs53576), and parental divorce status; the three 2-way

interactions of Sex × parental divorce status, sex × OXTR, and OXTR × parental divorce status; and the three-way interaction of Sex × parental divorce status × OXTR. Because of the smaller sample size to evaluate G × E interactions, bootstrapping was applied using the Mersenne twister (Matsumoto and Nishimura, 1998) and 1,000 bootstrap samples.

A second, highly similar prospective model was used to predict Wave 5, young adult depression, but depressive symptoms at Wave 4 were included in the specified model as an additional covariate to control for levels of adolescent depression. By including Wave 4 depression, a subtle but important distinction was made in that the model now predicted *change* in depression from adolescence to young adulthood rather than just prospectively predicting young adult levels of depression. This distinction is important because some studies have indicated high levels of continuity between depression in adolescence and young adulthood (Kandel and Davies, 1986; Cuijpers and Smit, 2004); without controlling for depression in adolescence, it would be difficult to know if the hypothesized G × E interaction existed in adolescence to impact depression and was then carried forward into young adulthood, or if it appeared to impact *changes* in depression from adolescence to young adulthood. Support for either of the two specified models would be valuable to the literature, but the interpretation would differ if the first model supported the G × E interaction but the second model did not. Hence, by specifying and evaluating both models a more rigorous evaluation of the hypothesized G × E interaction was facilitated. The statistical significance level of each model was provided, as well as the statistical significance of individual parameters and an overall model estimate of the variance accounted for (i.e., an adjusted R^2-value).

Results

Zero-order correlations and descriptive statistics for the sample are provided in **Table 1**. The sex distribution was females (n = 201; 59.1%) and males (n = 139; 40.9%), and 30 (8.8%) participants reported parental divorce status during adolescence. In bivariate comparisons via contingency tables, the distribution of the OXTR genotype did not differ significantly across sex groups [χ^2 (1) = 0.22, p = 0.66] or divorce status [χ^2 (1) = 1.95, p = 0.16], and sex group did not differ significantly across divorce status [χ^2 (1) = 0.11, p = 0.94].

The findings of the first regression model are summarized in **Table 2**. The overall model was statistically significant at $p < 0.10$ and young adult depression was significantly predicted by the hypothesized three-way interaction of Sex × OXTR × Parental divorce status. To facilitate the interpretation of this three-way interaction, the adjusted means of subgroups defined by sex, parental divorce status and OXTR genotype were plotted (**Figure 1**, top). Among males who experienced parental divorce, the proposed risk allele (GG) was associated with slightly lower depressive symptoms compared to the AA/AG genotype, but the difference was not significant [Cohen's d = –0.17; $t(330)$ = –1.54, p = 0.12]. By contrast, for females who experienced parental divorce, the proposed risk allele (GG) was related to significantly elevated CES-D depression scores from 9 to 21 [Cohen's d = 0.32; $t(330)$ = 2.95, $p < 0.01$]. No other differences between the subgroups emerged. These findings support the hypothesized three-way interaction in the prediction of depressive symptoms in young adulthood.

The findings of the second regression model that included Wave 4 depression as a covariate, are summarized in **Table 3**. The overall model was statistically significant and again young adult depression was significantly predicted by the hypothesized three-way interaction of Sex × OXTR × Parental divorce status. Adjusted means for all subgroups are depicted in **Figure 1** (bottom). Findings for this second model largely paralleled those of the first model. Among males who experienced parental divorce, the proposed risk allele (GG) was associated with lower depression scores, but the difference was not significant [Cohen's d = –0.15; $t(329)$ = –1.36, p = 0.18]. By contrast, the proposed risk allele (GG) was linked with higher levels of depression among females who had experienced parental divorce compared to the AA/AG allele carriers, with mean CESD-depression scores of 17 vs. 8 [Cohen's d = 0.26; $t(330)$ = 2.33, $p < 0.05$]. No other subgroups differed. These findings support the hypothesized three-way interaction in

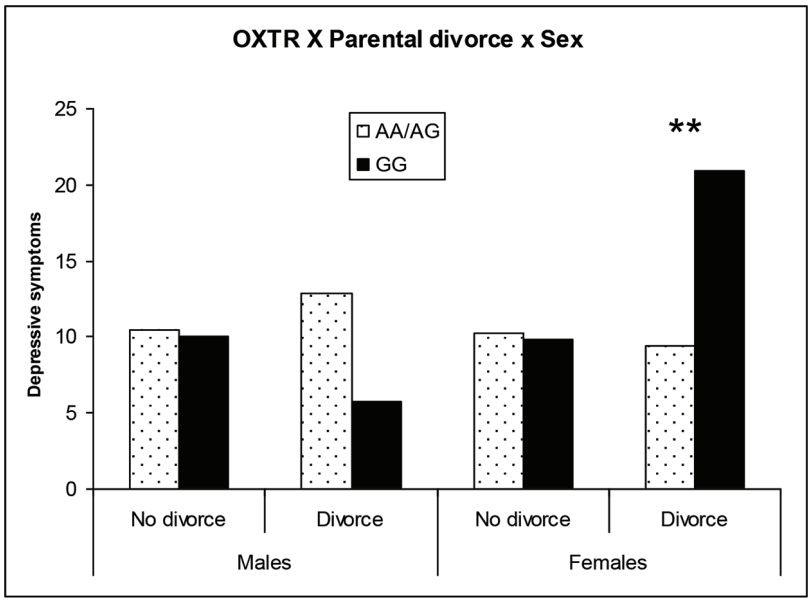
TABLE 1 | Correlations, means, and SD of demographic variables, divorce status, oxytocin, and depression (N = 340).

Variable	1	2	3	4	5	6	7
(1) Parent education	1.00						
(2) Family income	0.29	1.00					
(3) Gender (1 = M; 2 = F)	0.01	0.02	1.00				
(4) Divorce (0 = no; 1 = yes)	–0.01	–0.02	0.01	1.00			
(5) Oxytocin (0 = AA/AG; 1 = GG)	0.02	–0.01	–0.03	–0.08	1.00		
(6) W4 depression	0.12	–0.06	0.06	0.15	–0-.04	1.00	
(7) W5 depression	–0.06	–0.03	0.01	0.06	–0.02	0.33	1.00
M	2.88	6.42	1.59	0.09	0.49	15.02	10.29
SD	0.82	1.87	0.49	0.28	0.50	10.15	7.87

TABLE 2 | Bootstrapped regression model findings predicting depression in young adulthood (Wave 5)[1].

Source	B	Bias	*SE*	Significance
Intercept	11.70	–0.04	2.36	0.001
Parental education	–0.30	0.01	0.50	0.552
Family income	–0.02	–0.01	0.19	0.931
Sex (Female)	–0.21	0.07	1.18	0.859
Oxytocin (OXTR; GG)	–0.43	0.20	3.01	0.905
Divorce status	5.57	0.24	5.46	0.290
Sex*Divorce	–3.22	–0.20	3.50	0.331
Sex*OXTR	–0.03	–0.15	1.81	0.989
OXTR*Divorce	–25.19	–0.10	9.87	0.005
Sex*OXTR*Divorce	18.60	0.19	7.19	0.006

[1] *The F-statistic was 1.69, p = 0.089 and the adjusted R^2-value was 0.044.*



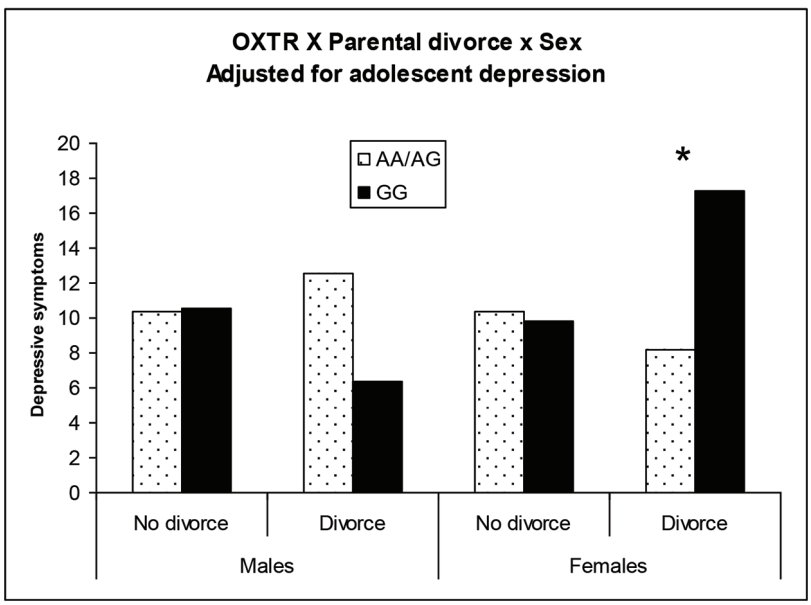


FIGURE 1 | Plot of significant OXTR × Sex × Parental divorce during adolescence interaction on young adult depression for males and females without and with adjustment for adolescent depression. *$p < 0.05$; **$p < 0.01$.

the prediction of change in depression from adolescence to young adulthood, although the effect sizes were of small magnitude.

Discussion

The findings of this study supported the hypothesized three-way interaction between sex, OXTR, and a significant adolescent stressor—parental divorce. Females with the GG risk allele manifested significant increases in depressive symptoms in young adulthood if parental divorce had occurred for them during adolescence; this relationship was not indicated for males. The findings were robust across both the prospective follow-up design in which adolescent depression was not controlled, as well as a longitudinal autoregressive change model in which adolescent depression was controlled, thereby supporting the interpretation that the three-way interaction predicted change in depressive symptoms from adolescence to young adulthood.

These findings contribute to the literature on moderators of the relationships between parental divorce and offspring depression in two ways (Booth and Amato, 2001; Lansford,

TABLE 3 | Bootstrapped regression model findings predicting depression in young adulthood (Wave 5) controlling for adolescent depression (Wave 4)[1].

Source	B	Bias	*SE*	Significance
Intercept	8.21	–0.02	2.26	0.002
Parental education	–0.80	0.02	0.49	0.098
Family income	0.12	–0.01	0.20	0.540
Sex (Female)	–0.02	0.03	1.10	0.990
Oxytocin (OXTR; GG)	0.88	0.18	2.93	0.777
Divorce status	6.48	0.18	5.07	0.188
W4 depression	0.25	0.01	0.05	0.001
Sex*Divorce	–4.32	–0.17	3.25	0.169
Sex*OXTR	–0.71	–0.13	1.75	0.685
OXTR*Divorce	–22.34	–0.11	8.58	0.004
Sex*OXTR*Divorce	–15.99	0.18	6.61	0.010

[1] *The F-statistic was 5.27, $p < 0.000$ and the adjusted R^2-value was 0.112.*

2009). First, they identify a molecular genetic factor, the OXTR polymorphism rs53576, as a statistically significant modifier of the parental divorce-offspring adjustment relationship, thereby opening-up new avenues of research that include genetic markers along with other pre- and post-divorce psychological and social factors. Second, the findings highlight potential advantages of embedding possible G × E relationships for the impact of parental divorce within a developmental framework, in this instance combining developmental studies of significant events/process (e.g., sex role development, sex differences in depression) in adolescence with findings of consilience in genetics, infrahuman studies, and cognitive neuroscience (Bakermans-Kranenburg and van IJzendoorn, 2008; Buchheim et al., 2009; Meyer-Lindenberg et al., 2011). Further investigation of this issue using the evolutionary perspective of Del Giudice (2014) are also merited, as it provides a cross-level, integrative approach.

The specific findings of this study for OXTR are most consistent with the findings of Costa et al. (2009), Bradley et al. (2011), and Sturge-Apple et al. (2012). We reported that OXTR interacted with a family stressor, parental divorce, during adolescence, to predict depression in young adulthood (differentially for males and females). Sturge-Apple et al. (2012) reported that OXTR interacted with a family stressor, interparental conflict, and maternal sensitivity to toddlers as their outcome, and a similar G × E interaction was supported. Likewise, Costa et al. (2009) reported that the GG allele combination was associated with the highest levels of separation anxiety and insecure attachments among adults with depression. Our study does not resolve the mixed findings that have been reported in the OXT-social affiliative behaviors literature (Bakermans-Kranenburg and van IJzendoorn, 2008; Costa et al., 2009), but does provide additional support for considering G × E interactions, possible sex differences (Tost et al., 2010), and expanding the age range for developmental studies beyond infancy and childhood.

A major limitation of candidate gene studies has been the failure of replication, due in part, to underpowered sample sizes and to a failure to correct for multiple hypothesis testing (Duncan and Keller, 2011; Duncan et al., 2014; Manuck and McCaffery, 2014). An underpowered sample size is a limitation of this study, although the sample sizes and cross-validation samples of tens of thousands recommended for GWAS are highly improbable for research designs (e.g., clinical trials, neuroimaging studies, pharmacogenetic studies) and complex phenotypes of interest in psychology, psychiatry, and the behavioral sciences. Furthermore, it often is challenging to replicate developmental G × E studies or to conduct G × E meta-analyses because studies would require similar samples, with similar measures, and similar time-points (or waves of assessment). Nevertheless, replication studies are clearly needed to confirm and extend the findings of this, and other behavioral science studies. The limitations of candidate gene studies, such as the current one, can also be reduced by using extant empirical findings and theories, including GWAS findings and developmental and evolutionary theories, to guide hypotheses formation and to form polygenic indexes. Issues related to small sample size, relative to other sampling strategies (e.g., GWAS), can also be attenuated by selecting a specific genotype or small set of genotypes (e.g., polygenic indexes) rather than using exploratory data-mining procedures that require stringent corrections to the nominal alpha level for statistical testing. This study also has other limitations such as sample restrictions with respect to the representation of diverse ethnic groups and to a broader socioeconomic range. It also does not contain variables that would facilitate the evaluation of the intervening mediators and mechanisms that may account for the obtained relationships, and other risk factors for young adult depression were not included in the models. Despite these limitations, the findings do provide insight into how G × E relationships, embedded within a well-supported research literature, can advance our understanding of parental divorce and young adult depression.

Author Contributions

Both authors (MW and SM) made substantial contributions to the conception and design of this article. MW conducted most of the data analyses and both authors contributed to the interpretation of findings. Both authors participated in drafting and revising the work for intellectual content, and both provided final approval of the version to be published. The authors also agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Genetic sensitivity to emotional cues, racial discrimination and depressive symptoms among African–American adolescent females

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Psychosocial stress, including stress resulting from racial discrimination (RD), has been associated with elevated depressive symptoms. However, individuals vary in their reactivity to stress, with some variability resulting from genetic differences. Specifically, genetic variation within the linked promoter region of the serotonin transporter gene (*5-HTTLPR*) is related to heightened reactivity to emotional environmental cues. Likewise, variations within this region may interact with stressful life events (e.g., discrimination) to influence depressive symptoms, but this has not been empirically examined in prior studies. The objective of this study was to examine whether variation in the *5-HTTLPR* gene interacts with RD to predict depressive symptoms among a sample of African–American adolescent females. Participants were 304 African–American adolescent females enrolled in a sexually transmitted disease prevention trial. Participants completed a baseline survey assessing psychosocial factors including RD (low vs. high) and depressive symptomatology (low vs. high) and provided a saliva sample for genotyping the risk polymorphism *5-HTTLPR* (*s* allele present vs. not present). In a logistic regression model adjusting for psychosocial correlates of depressive symptoms, an interaction between RD and *5-HTTLPR* group was significantly associated with depressive symptomatology (AOR = 3.79, 95% CI: 1.20–11.98, $p = 0.02$). Follow-up tests found that high RD was significantly associated with greater odds of high depressive symptoms only for participants with the *s* allele. RD and *5-HTTLPR* status interact to differentially impact depressive symptoms among African–American adolescent females. Efforts to decrease depression among minority youth should include interventions which address RD and strengthen factors (e.g., coping, emotion regulation, building support systems) which protect youth from the psychological costs of discrimination.

Keywords: racial discrimination, genetic sensitivity, depressive symptoms, adolescents, *5-HTTLPR*

Introduction

Across a wide range of health indicators, including mental health outcomes, dramatic racial and socioeconomic health disparities exist for African–American adolescents in the United States (Williams et al., 2010). A variety of factors have been examined to elucidate what drives these persistent disparities. A burgeoning body of literature indicates that racial discrimination (RD) may be a key factor associated with increased risk for many negative health outcomes, including both poorer physical (e.g., coronary artery calcification, sexually transmitted infections, and low birth-weight infants) and mental health outcomes (e.g., increased rates of internalizing and externalizing conditions; Lewis et al., 2006; Brondolo et al., 2008; Dominquez et al., 2008; Pascoe and Smart Richman, 2009; Williams and Mohammed, 2009; Rosenthal et al., 2014). Young African–American women in particular also experience high rates of depressive symptoms relative to their non-minority peers (Khan et al., 2009). For example, 19.3% of African–American adolescent women in the National Longitudinal Study of Adolescent Health endorsed recent and chronic depressive symptoms relative to 13.0% of White adolescent women (Khan et al., 2009). However, not everyone experiencing elevated stressors, including RD stress, evidences negative mental health outcomes. Thus, the primary purpose of this study was to examine the extent to which genetic variation moderates the association between RD and high levels of depressive symptomatology among a sample of adolescent African–American women. In other words, are some adolescent African–American women more affected than others by RD thereby resulting in higher levels of depressive symptoms?

Racial discrimination is defined as dominant group members' actions that have a differential and negative effect on subordinate racial/ethnic groups (Williams et al., 2003). Previous research has indicated that 91% of pre-adolescent African–Americans reported one or more experiences of race-related discrimination in their lifetime (Gibbons et al., 2004). Similarly, another study found that 77% of African–American youth reported experiencing one or more discriminatory events in the prior 3 months (Prelow et al., 2004). Specific to African–American female adolescents, Guthrie et al. (2002) found that 52% reported at least one exposure to RD in the past year (Guthrie et al., 2002). The Integrative Model for the Study of Developmental Competencies in Minority Children (Integrative Model) by Garcia Coll et al. (1996) proposes that individuals in American society are stratified based upon social position factors (e.g., race, social class, and gender), and social positions are influenced by RD. Because RD is embedded within American society it is a normative and chronic exposure for African–American children and adolescents (Garcia Coll et al., 1996). Thus, RD is a pervasive challenge in the lives of adolescent African–American females, and has been posited by Thoits (1991) and more recently, by Jones (2000) to be a stressor that, if internalized (i.e., internalized racism), threatens the central parts of an individual's identity, thereby adversely affecting one's mental health.

A growing body of research suggests that RD is especially harmful to the mental health of African–American youth. Several cross-sectional studies have demonstrated that experiences with RD are associated with lower self-esteem, increased anger, and increased anxiety and depressive symptoms among African–American adolescents (Prelow et al., 2004; Seaton et al., 2008; Gaylord-Harden and Cunningham, 2009). Many of these associations have been found in longitudinal studies as well. Specifically, racial discriminatory experiences are related to decreased self-esteem and increased conduct problems and depressive symptoms among African–American youth (Brody et al., 2006, 2011; Greene et al., 2006; Gibbons et al., 2007; Neblett et al., 2008; Estrada-Marinez et al., 2012).

Depression is one of the most common psychiatric issues affecting adolescents. At any given time, ∼15% of children and adolescents exhibit some symptoms of depression, while 5% of 9- to 17-year-olds meet the criteria for a major depressive disorder (Birmaher et al., 1996; Shaffer et al., 1996). Specific to adolescents, the incidence of depressive disorders markedly increases after puberty. Moreover, by 14 years of age, depressive disorders are more than twice as common in females as in males (Angoid et al., 1999). Female adolescents, a group disproportionately affected by depression, experience heightened interpersonal, relationship stress relative to their male peers (Rudolph, 2002; Hampel and Petermann, 2006; Teva et al., 2010). Added to this, African–American adolescents encounter greater chronic, contextual or environmental stressors relative to their non-minority peers (Copeland-Linder et al., 2011) such as RD (Clark et al., 1999; Sellers et al., 2006; Gaylord-Harden and Cunningham, 2009), among others. Thus for African–American young women in particular, elevated exposure to chronic psychosocial and environmental stressors such as interpersonal stress and RD have been posited as core constructs that may underlie increased vulnerability to depressive disorders and heightened depressive symptoms (Estrada-Marinez et al., 2012). Despite this well-documented association between RD and increased depressive symptoms, surprisingly few studies have examined factors which moderate the association between RD and depressive symptomotology among African–American adolescent females (Seaton et al., 2014), and few, to our knowledge, have taken into account other common sources of stress also associated with depressive symptoms among young women (e.g., interpersonal stress and abuse history; Estrada-Marinez et al., 2012).

Neuroscientists have noted that some individuals are particularly reactive to emotional environmental stimuli, such as exposure to racial discriminatory events. Functional magnetic resonance imaging (fMRI) studies suggest the amygdala, a region of the brain critical for emotional processing and especially important for detection and processing of anxiety and fear-related information, is affected by genetic variability in the promoter of the *5-HTT* gene (*5-HTTLPR;* Nordquist and Oreland, 2010). The *5-HTT* gene is a key regulator of serotonergic neurotransmission, localized to 17p13 and consisting of 14 exons and a single promoter. The common polymorphism in the promoter region results in two variants, a short and a long allele, with the short allele resulting in lower serotonin transporter availability. Although there has been continued debate about the extent to which *5-HTTLPR* moderates the association between

stress and depression (see Risch et al., 2009 and Karg et al., 2011 for recent meta-analyses on the topic), relative to those without a short allele, individuals with at least one copy of the short (*s*) allele of the *5-HTTLPR* who have also experienced stressful life events have been suggested to have higher rates of depressive disorders or depressive symptoms.

Aside from this conceptualization of the role of *5-HTTLPR* in emotion-related processing, other studies have found that individuals with at least one copy of the *s* allele of the *5-HTTLPR* have increased amygdala activation to fearful stimuli in facial expression recognition tasks and enhanced amygdala reactivity to punishment cues in the environment (Hariri et al., 2002, 2003; Battaglia et al., 2005; Hariri et al., 2006). Further, carriers of at least one copy of the *s* allele also display hyperactive amygdala response to non-emotional and neutral cues (Heinz et al., 2007; Munafo et al., 2008), direct preferential attention toward threat-related stimuli, and also have difficulty disengaging from such stimuli (Osinsky et al., 2008; Beevers et al., 2009).

Taken together, neuroscience research suggests that carrying the *s* allele may prompt enhanced emotional arousal to threatening and stressful environmental events, resulting in higher levels of depressive symptoms among those with high levels of stress exposure, particularly chronic environmental stressors (e.g., RD). In other words, these findings suggest that African–American adolescent females who experience high levels of RD AND who are also predisposed toward heightened emotional reactivity to environmental events by having at least one copy of the *s* allele of the *5-HTTLPR* should evidence greater levels of depressive symptoms than those without the genetic sensitivity or with lower levels of exposure to RD.

The purpose of the present study was to examine whether variation in the *5-HTTLPR* gene interacts with level of RD to predict depressive symptoms among a sample of adolescent African–American females, while accounting for relevant factors such as coping and social support and stressors (abuse history and interpersonal stress). We hypothesized a gene by environment (G × E) interaction effect in which the association between high levels of RD and high levels of depressive symptoms would be more evident among those individuals carrying at least one copy of the *s* allele of the *5-HTTLPR* polymorphism.

Materials and Methods

Description of Parent Study Recruitment

This study is a secondary analysis of data collected as part of a randomized STD/HIV prevention trial specially designed and tailored for adolescent African–American female youth (HORIZONS; DiClemente et al., 2014). From July 2005 to June 2007 African–American adolescent females were recruited from three reproductive health clinics (an adolescent reproductive health clinic in a public hospital, a community located reproductive health clinic, and a county health department STD clinic in Atlanta, GA) to participate in an STD/HIV prevention trial. The adolescent clinic serves ∼2000 clients annually, the majority of clients attending the clinic are ages 16–17, over 90% of clinic attendees are sexually active and the vast majority (over 85%) is African-American. The community-located clinic serves ∼3,300 clients per year; around 80% of female clients are 14-29 years, and ∼50% are African–American. The county health department sexually transmitted disease (STD) clinic serves predominately African–American clients (over 80%), with ∼5,000 adolescent visits to their STD/HIV/AIDS Program annually. The median age of the teens attending the clinic for STD/HIV-related care was about 16 years, and females outnumber males by about two to one.

The purpose of the trial was to assess whether a supplemental treatment delivered after intervention workshop participation (via phone calls) enhanced maintenance of a modified efficacious STD/HIV behavioral intervention (HORIZONS; DiClemente et al., 2014). A young African–American woman recruiter approached adolescents in the clinic waiting area, described the study, solicited participation, and assessed eligibility. Eligibility criteria included self-identifying as African–American, being 14–20 years of age, and reporting at least one instance in the past 6 months of vaginal intercourse without a condom. Young women were excluded from the study if they were married, pregnant, or attempting to become pregnant. Those meeting inclusion criteria and interested in participating returned to the clinic to complete informed consent procedures, baseline assessments, and randomization to trial conditions. Written informed consent was obtained from all young women. Parental consent was waived for those younger than 18 due to the confidential nature of clinic services. Of the eligible individuals, 94% ($N = 701$) enrolled in the study, completed baseline assessments and were randomized to study conditions. Participants were compensated $75 for the baseline visit. The Emory University Institutional Review Board approved all study protocols.

Procedures and Measures Relevant for the Current Study

As part of the parent study's procedures, participants completed an audio computer assisted self-interviews (ACASI) at baseline, prior to randomization and intervention participation. The baseline ACASI data allowed for assessment of all variables included in this study such as sociodemographics, coping, social support, abuse history, interpersonal stress, RD, and depressive symptoms. In addition to the baseline ACASI survey completed as part of the parent study, this analysis reports on data from the 304 participants who also consented and provided a saliva sample for DNA analysis[1].

Primary Outcome Variable

Depressive symptoms

Depressive symptoms were assessed with a very brief, eight-item version of the Center for Epidemiological Studies-Depression scale (Melchior et al., 1993). The CES-D assesses the presence of

[1]The DNA sample collection was an addition to the main trail's data collection. Because of this, not every participant was invited to provide a sample if they: (1) had already completed the trial, or (2) did not return for the 24 month follow-up assessment when the sample collection occurred. 363 were asked to provide a sample, and only 31 declined.

depressive symptoms in the past 7 days and has been shown to be a valid measure of depressive symptoms in African–Americans (Radloff, 1991). A total score was first calculated with higher scores indicative of higher depressive symptom levels; the range of possible scores is 8–32. Scores above 15 suggest clinically elevated depressive symptom levels (coded as 1) relative to those with scores below 15 (coded as 0). Cronbach's α, a measure of the scale's internal consistency, was 0.91.

Primary Predicator Variable

Racial discrimination

A 13-item revised version of the Schedule for Racist Events scale (SRE; Landrine and Klonoff, 1996) was used to measure RD. This revised version has been extensively used by other researchers among adolescent and young adult samples (Simons et al., 2003, 2006; Gibbons et al., 2004; Brody et al., 2006). The revised SRE assessed the frequency during the past year, ranging from 1 (never) to 4 (several times), with which the participant experienced specific discriminatory behaviors such as racially based slurs or insults, disrespectful treatment from community members, physical threats, and false accusations from law officials or business employees. The mean score (mean = 20.36, SD = 6.93; median = 20; possible range = 13–52; observed range = 13–45) was used to split the sample into those reporting higher than the average levels of discrimination experiences (scores greater than 20 = 1 "high discrimination") and those reporting average or lower discrimination experiences (scores 20 or less = 0 "low discrimination"). Cronbach's α was 0.90.

Control Variables

Sociodemographic measures

Age was assessed by asking, "How old are you (in years)?" Also, clinic location was included as a control variable as participants were recruited from three downtown Atlanta reproductive health/STD clinics; each serving slightly different populations in regards to SES and education.

Psychosocial Correlates

Interpersonal stress

A 13-item modified version of the African–American Women's Stress Scale (Watts-Jones, 1990) was used to measure interpersonal or family stressors. Questions assess the amount of stress an individual feels in various interpersonal relationships or contexts (e.g., relationships with family, partner not being faithful, and isolation from family). Cronbach's α for the scale was 0.87.

History of abuse

Abuse was conceptualized as an index comprising four forms of abuse; emotional, physical, forced vaginal sex or forced anal sex. Abuse history was assessed by asking four questions, "Have you ever been emotionally abused (threatened or called names)," Have you ever been physically abused (hit, kicked, slapped, punched)?", "Has anyone ever forced you to have vaginal sex when you didn't want to?", and "Has anyone ever forced you to have anal sex when you didn't want to?". Response choices were yes (1) and no (0). Consistent with the definition used in national surveillance studies (Leeb et al., 2008), a dichotomous composite variable was created in which participants who indicated yes on any of the four items were determined to have a history of abuse, and those who answered no on all items were determined to have no history of abuse.

Coping

A 14-item modified version of the COPE scale was used to assess reliance on avoidance-based coping (Carver et al., 1989). Examples of coping behaviors queried were, "I act as though it hasn't even happened," or "I admit to myself that I can't deal with it, and quit trying." Higher scores indicate more reliance on avoidance-based coping. Cronbach's α was 0.78.

Social support

Social support was assessed with a 12-item scale (Zimet et al., 1988). Responses were coded so that higher scores reflected higher levels of perceived social support by the adolescent. An example item is, "I get the emotional help and support I need from my family." Cronbach's α was 0.90.

Genotyping

DNA was obtained using Oragene^{TM} DNA kits (Genetek; Calgary, AB, Canada). Participants rinsed their mouths with tap water, and then deposited 4 ml of saliva in the Oragene sample vial. The vial was sealed, inverted, and shipped via courier to a central laboratory in Iowa City, where samples were prepared according to the manufacturer's specifications. Genotype at *5-HTTLPR* was determined for each sample as previously described (Bradley et al., 2005). Of the sample, 9.2% were homozygous for the short allele (*ss)*, 34.2% were heterozygous (*sl*), and 56.6% were homozygous for the long allele (*ll*). Consistent with prior research (Hariri et al., 2005), genotyping results were used to form two groups of participants: those homozygous for the long allele ($n = 172$) and those with either 1 or 2 copies of the short allele ($n = 132$). Among the 332 participants who provided a saliva sample, 5.12% ($n = 17$) had a "very long" variant of *5-HTTLPR*. Because the activity of this variant on the hypothesized associations has not been well characterized, these youths were excluded from the data analyses.

Data Analysis Plan

All analyses were limited to the 304 main trial participants who, in addition to the baseline assessment, consented and provided a valid saliva sample for DNA analysis. Descriptive statistics summarized study variables. In addition, bivariate analyses examined associations between control variables, *5-HTTLPR* group (i.e., *s* allele group vs. *ll* allele group), psychosocial factors associated with managing stressful experiences (i.e., social support and coping), RD group (i.e., low vs. high) and depressive symptoms (i.e., low vs. high). Associations were assessed using Pearson's correlations and Chi-square analyses. Variables significant at the $p \leq 0.10$ in bivariate analyses were entered into a multivariable hierarchical logistic regression predicting high depressive symptoms (Hosmer and Lemeshow, 2000), controlling for age and clinic. In the first step, psychosocial correlates were entered into the model. In the second step, RD

and *5-HTTLPR* were entered into the model. In the final step, to explore whether the association between discrimination and depressive symptoms differed as a function of *5-HTTLPR* group, an interaction between discrimination group and *5-HTTLPR* group was entered in at this step of the regression model.

Results

Sample Description

Descriptive statistics for all measures are presented in **Table 1**. The majority was still in high-school or had only completed some high-school at enrollment (53.9%). Many reported living with their mother only (42.9%), and approximately a quarter had a job for which they were paid. Many of the participants were recruited from a county health department STD clinic ($n = 154$), others were recruited from a reproductive health clinic ($n = 119$), and the remaining participants were recruited from an adolescent reproductive health clinic in a public hospital ($n = 31$). Of the 304 participants in this study 82.2% ($n = 250$) endorsed experiencing a least one of the 13 forms of RD on in the past year.

Bivariate Associations Among Study Variables

Pearson correlations or Chi-square tests were conducted among potential control variables, the primary predictor variable (RD), 5-HTTLPR status and depressive symptoms. Only significant ($p \leq 0.05$), or marginally significant ($p \leq 0.10$), associations are described. Specific to the control variables, participant age was positively correlated with RD ($r = 0.15$, $p = 0.01$). Also, participants recruited from the health department STD clinic were more likely to report high depressive symptoms (40.9%) than those recruited from the adolescent clinic (32.3%), and the reproductive health clinic (27.7%); $\chi^2 = 5.24$, $p = 0.07$. Among the psychosocial factors, interpersonal stress ($r = 0.40$, $p < 0.001$), history of abuse ($r = 0.32$, $p < 0.001$), and avoidance-based coping ($r = 0.27$, $p < 0.001$) were each positively correlated with higher depressive symptoms. Participants with high RD were more likely (45.7%) to report high depressive symptoms compared to the those with low discrimination (29.4%); $\chi^2 = 19.63$, $p < 0.001$. However, participants *5-HTTLPR* status was not significantly related to depressive symptoms ($r = 0.03$, $p = 0.67$), nor were participants with an *s* allele more likely to report higher RD (43.2%) than those in the *ll* allele group (40.7%); $\chi^2 = 0.19$, $p = 0.66$.

TABLE 1 | Descriptive statistics of the study sample on study variables (*N* = 304).

	Mean	SD
Sociodemographic		
Age	18.09	1.40
Possible psychosocial control variables		
Interpersonal stress	28.41	13.51
Coping	18.94	4.64
Social support	35.99	5.80
Abuse history (frequency/%)	197	64.8
Primary predictor variable	**Frequency**	**%**
High racial discrimination[a]	127	41.8
Outcome		
High depressive symptoms[a]	106	34.9

[a]*Rather than means and SD, frequency and percent is displayed.*

Multivariable Hierarchical Logistic Regression Predicting Level of Depression Symptoms

Overall, we found that the three step model including the interaction term was significant (see **Table 2**). The interaction between RD and 5-*HTTLPR* group was significantly associated with the probability of being in the high depressive symptom group above and beyond the psychosocial factors. In order to interpret the interaction effect, separate multivariable logistic regression models for level of depressive symptoms were conducted for those possessing one or two copies of the *s* allele and those with the *ll* allele (see **Table 3**). For both those with the *s* and the *ll* genotypes, having higher interpersonal stress and a history or abuse were associated with higher odds for elevated depressive symptoms. However, RD was associated with higher odds of elevated depressive symptoms only among those with the *s* allele, but engagement in avoidance-based coping was associated with higher odds for elevated depressive symptoms among the *ll*-genotype.

An additional follow-up test was conducted to further examine the proposed differential susceptibility hypothesis, whereby those with the *s* allele are more sensitive toward and responsive to their environment. A layered Chi-Square test was conducted, with separate Chi-Square tests run by *5-HTTLPR* group (*s* allele group and the *ll* allele group) to determine the association between level of RD (low vs. high) and level of depressive symptoms (low vs. high) for each genetic group (see **Figure 1**). For those youth in the s allele group ($n = 132$), participants with high levels of discrimination experiences were significantly more likely to report high levels of depressive symptoms (45.6%) than those with low levels of discrimination experiences (22.7%); $\chi^2 = 7.77$, $p = 0.005$. In contrast, among those in the *ll* allele group ($n = 172$), participants with high levels of discrimination experiences were not more likely to report high levels of depressive symptoms (41.4%) than those with low levels of discrimination experiences (33.3%); $\chi^2 = 1.17$, $p = 0.28$.

Discussion

Similar to previous reports of RD levels in the U.S., almost all participants in this African–American adolescent female sample reported experiencing RD in the prior year, with ∼41% indicating higher than average experiences of discrimination. This sample also showed high levels of depressive symptoms, with ∼35% reporting levels of depressive symptoms that are potentially clinically significant. In addition to other stressors (interpersonal stress and abuse histories), as expected (Prelow et al., 2004; Seaton et al., 2008; Gaylord-Harden and Cunningham, 2009), RD was significantly associated with depressive symptoms among this all female sample. However, results demonstrated a moderating role of *5-HTTLPR* genotype status in this outcome. Among this

TABLE 2 | Multivariable hierarchical logistic regression predicting level of depressive symptoms.

Predictors	β	SE	Odds ratio	95% CI		p
				Lower	Upper	
Step 1						
Psychosocial correlates						
Interpersonal stress	0.06	0.01	1.06	1.04	1.08	0.001
Coping	0.11	0.03	1.12	1.05	1.20	0.001
Abuse history	1.15	0.36	3.15	1.56	6.35	0.001
Step 2						
5-HTTLPR group	−0.72	0.40	0.49	0.22	1.06	0.071
Racial discrimination group	−0.28	0.39	0.76	0.35	1.66	0.486
Step 3						
Discrimination X *5-HTTLPR* group	1.33	0.58	3.79	1.20	11.98	0.023
Step 1 χ^2=	82.15					0.001
Step 2 χ^2=	1.16					0.561
Step 3 χ^2 =	5.31					0.021
Overall modelχ^2 =	88.61					0.001

Age and clinic were controlled for in regression.

TABLE 3 | Multivariable logistic regressions predicting level of depressive symptoms, separately for each *5-HTTLPR* group.

Predictors	β	SE	Odds ratio	95% CI		p
				Lower	Upper	
***5-HTTLPR s* allele group**						
Primary predictor						
Racial discrimination group	0.91	0.45	2.48	1.02	6.05	0.045
Psychosocial factors						
Interpersonal stress	0.06	0.02	1.06	1.02	1.10	0.002
Coping	0.05	0.05	1.05	0.95	1.16	0.358
Abuse history	1.38	0.55	3.93	1.33	11.64	0.014
Overall χ^2 =	36.05					0.001
***5-HTTLPR ll* allele group**						
Primary predictor						
Racial discrimination group	−0.20	0.43	0.82	0.35	1.90	0.637
Psychosocial factors						
Interpersonal stress	0.06	0.02	1.06	1.03	1.09	0.001
Coping	0.16	0.05	1.18	1.08	1.29	0.001
Abuse history	0.96	0.49	2.61	1.01	6.76	0.048
Overall χ^2 =	55.55					0.001

Age and clinic were controlled for in regressions.

sample of young African–American women, high exposure to RD was associated with greater odds of elevated depressive symptoms only for young women with the *s* allele, even when accounting for multiple sources of stress and psychosocial factors, while the link between RD and depressive symptoms was attenuated for those with the *ll* genotype. Among individuals with this genotype, greater reliance on avoidance-based coping behaviors were instead significantly associated with likelihood of elevated depression symptoms.

Adolescence is a period of great developmental change (Crosby et al., 2009) characterized by increasing levels of psychosocial stressors (Compas, 1987). For young women especially, adolescence is a time when many may experience depressive symptoms for the first time (Angoid et al., 1999). African–American adolescents may also encounter additional chronic, contextual or environmental stressors relative to their non-minority peers (Copeland-Linder et al., 2011; Estrada-Marinez et al., 2012). This stressful exposure includes RD, which has been specifically associated with increased risk for elevated depressive symptoms particularly among young women (Clark et al., 1999; Sellers et al., 2006; Gaylord-Harden and Cunningham, 2009). The genetic marker of *5-HHTLPR* as indicated through the neuroscience findings seems to be especially relevant for the detection of fear or threat specific

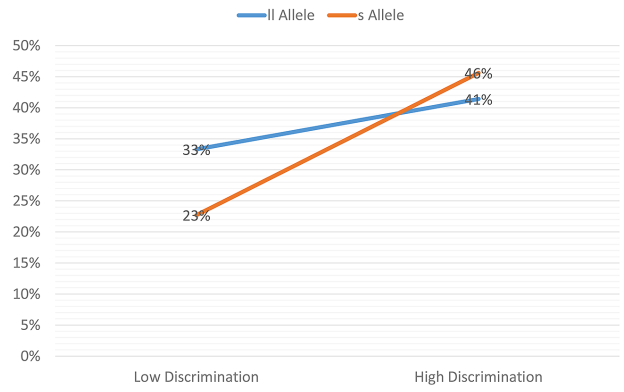

FIGURE 1 | Percent reporting high depressive symptoms: interaction between discrimination and *5-HTTLPR*.

stimuli in the environment (particularly in facial cues). The neuroscience findings that individuals with the *s* allele seem to be hyperaroused and hypersensitive to cues of fear/threat make this marker particularly important in the context of some stressors (like racial discriminatory behaviors such as physical threats, slurs, insults, and it is also relevant for individuals with histories of physical or sexual abuse as well), but it may not be as relevant in other stressful contexts that may not contain the same sort of emotional triggers detected by monitoring others emotional states, such as interpersonal stressors like someone owing you money, or minor daily hassles captured in our interpersonal stress scale. However, this speculation warrants further examination.

In accordance with the differential susceptibility theories (Belsky et al., 2007), we found a link between exposure to high levels of RD and depressive symptoms among youth who carried the one of two copies of the *s* allele of the *5-HTTLPR*. This finding is consistent with neuroimaging studies and prior G × E research involving the *5-HTTLPR* such that female youth who carry the short allele, because of their genetic make-up, may be more reactive to emotional or threating social cues in their environments (e.g., being disrespectfully treated by community members) and are therefore more negatively impacted by experiences of RD than those with the *ll* genotype. The finding that carrying two copies of the long allele may confer protection from depressive symptoms when they experience high levels of discrimination is relevant to research on youth resilience.

The resilience literature has addressed potential reasons why some youth who experience adverse experiences, including exposures to chronic stressors, do not succumb to their negative effects (Luthar, 2006). The present results support recent findings suggesting that genetic status may also contribute to resilience (Rutter and Silberg, 2002; Moffitt et al., 2006; Kim-Cohen and Gold, 2009; Brody et al., 2011). Particularly, the finding that carrying two *l* alleles attenuates the association between discrimination and depressive symptoms suggests a possible emotional self-regulatory mechanism by which genotype contributes to the down regulation of emotions resulting from discriminatory experiences (Simons et al., 2003, 2006; Brody et al., 2011), but this potential mechanistic pathway requires further investigation. Further, the potentially protective effects of the *ll* allele may not be generalizable to all youth, or under all contexts. Similar to Estrada-Marinez et al.'s (2012) findings that not all stressors are equally impactful on externalizing and internalizing outcomes, our results also suggest that young women with the *ll* allele may be resilient in some areas but experience distress in others, such as when they experience certain aspects of interpersonal relationships that may not be perceived as threatening yet cause stress.

Our findings also are in line with Belsky et al. (2007) differential susceptibility hypothesis in which gene-specific variants are speculated to render individuals more susceptible to their surrounding environments, whether those be "good" or "bad" environments. Specifically, we observed that young women who carried the *s* allele, in low discriminatory contexts reported lower levels of depressive symptoms than did *ll* allele carriers (see **Figure 1**), thereby supporting the hypothesis of differential susceptibility. However, we know very little about other positive or protective attributes of these young women's environments, but this would be an important avenue for further investigation, especially as it may shed light on potential protective factors that could serve as intervention opportunities to decrease adverse mental health outcomes among those adolescent women exposed to high levels of discrimination.

From an intervention perspective, the results suggest that young African–American women seeking sexual health services would benefit from additional resources and skills training to address depressive symptoms and cope with chronic, pervasive stressors including experiences of RD. For example, multiple health-related intervention approaches may benefit from inclusion of content to improve coping and self-management strategies. Specifically, it may be especially beneficial given the high rates of stress (whether from interpersonal relationships or from experiences of discrimination) that health promotion programs for adolescent and young adult African–American females in general include mental-health specific components, such as teaching developmentally appropriate stress-coping skills and cognitive behavioral management skills (Antoni et al., 2001) mindfulness training that would help youth learn relaxation techniques for managing uncontrollable stressors (Bishop, 2002), and strategies for building and accessing social support systems. These components could be integrated into existing STD prevention interventions in the case of this study, or other health promotion programs, to address likely unmet mental health needs of the youth who are experiencing high levels of discrimination or other chronic stressors. Importantly, for some youth (those with the *s* allele) who are particularly reactive to environmental cues of threat, other clinical techniques (e.g., exposure therapy approaches) may be useful to reduce threat sensitivity.

Limitations

This study is not without limitations. First, the sample consisted of adolescents who were seeking services at sexual health clinics, who met eligibility criteria for the parent study, and

who attended the follow-up visit when the genetic sample was collected. Therefore results may not generalize to individuals who do not access similar clinics, who would not meet the eligibility criteria, which included having recent unprotected sex, or who are not likely to return for follow-up. Future research should include a broader sample of youth, as well as include males to extend or replicate these findings. Also, the data employed in this study were only from participants who returned at the 24-month follow-up assessment and provided a saliva sample. It is possible that returning participants may have differed in meaningful ways from those who did not return for follow-up, but we have no way to formally examine this possibility. However, analyses of baseline socioeconomic indicate no significant differences between those who returned for follow-up and those who did not. Additionally, participants who provided DNA samples may have differed from participants who did not provide a specimen. However, we experienced a high rate of participation for the DNA saliva collection (92%), and a comparison of baseline characteristics indicates no observed differences in sociodemographics, psychosocial variables, or behavioral outcomes. Finally, the self-report data are cross-sectional, making it difficult to assess causal relationships, although a strength is that we directly assessed RD in our study.

Conclusion

Adolescence is a period of life characterized by developmental change and increasing levels of psychosocial stressors (Compas, 1987). For African–Americans, many will encounter additional chronic, contextual or environmental stressors such as exposure to RD relative to their non-minority peers (Copeland-Linder et al., 2011; Estrada-Marinez et al., 2012). However, consistent with neuroimaging studies and prior G × E research involving the *5-HTTLPR*, for some individuals, because of their genetic make-up, they are particularly susceptible to negative psychological consequences resulting from high exposure to RD than others. Given the high rates of depressive symptoms coupled with high number of stressors reported among adolescent African–American (whether from abuse experiences, interpersonal relationships or from experiences of discrimination) it may be advantageous for health promotion programs targeting adolescent and young adult African–American females in general to include mental-health specific components, such as teaching developmentally appropriate stress management and cognitive behavioral skills.

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Integrating basic research with prevention/intervention to reduce risky substance use among college students

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Too often basic research on etiological processes that contribute to substance use outcomes is disconnected from efforts to develop prevention and intervention programming. Substance use on college campuses is an area of concern where translational efforts that bring together basic scientists and prevention/intervention practitioners have potential for high impact. We describe an effort at a large, public, urban university in the United States to bring together researchers across the campus with expertise in college behavioral health with university administration and health/wellness practitioners to address college student substance use and mental health. The project "Spit for Science" examines how genetic and environmental influences contribute to behavioral health outcomes across the college years. We argue that findings coming out of basic research can be used to develop more tailored prevention and intervention programming that incorporates both biologically and psychosocially influenced risk factors. Examples of personalized programming suggest this may be a fruitful way to advance the field and reduce risky substance use.

Keywords: spit for science, prevention, intervention, substance use, college students

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Translational Research in Psychological Science: Obstacles and Opportunities

Translational research has been a priority of the National Institutes of Health for many years (Butler, 2008); however, a chasm remains between basic research and the application of that research to alleviate illness. This gap is often discussed within the realm of medicine with respect to the application of basic, bench-side research to patient treatments ("bench to bedside"). However, it is no less relevant in the field of psychology, where basic psychological scientists often conduct research with little connection to researchers or practitioners involved in the more applied prevention and intervention work that their research informs. Basic researchers, applied researchers, and practitioners often attend their own conferences and publish in their own journals, which hampers by-directional feedback that could be informative for both sides.

In the fall of 2011, we launched a university-wide research project ("Spit for Science"; Dick et al., 2014) focused on genetic and environmental influences on substance use and mental health among college students. This project provided the impetus for an effort to bring together relevant individuals

across the university concerned about student substance use. Here we provide an overview of this university initiative and our perspective on how developing connections between basic researchers and applied practitioners can be mutually beneficial. We argue that the university setting is a tremendous opportunity to forge these translational relationships.

College Student Substance Use: The Need for Improved Approaches

Risky substance use among college students is widespread, with 39% of students reporting that they are binge drinkers (Substance Abuse and Mental Health Services Administration, 2012), and 36% of students reporting illicit drug use in the past year (Kilmer and Geisner, 2013). Nearly half (47%) of all students meet criteria for an alcohol or marijuana use disorder at least once in the first 3 years of college (Caldiera et al., 2009). Substance use is associated with a number of adverse consequences, including decreased academic performance and graduation rates (Kitzrow, 2003), as well as unwanted sexual encounters, legal consequences, assault, injury, suicide, and death (Wechsler et al., 2002; Hingson et al., 2009; Arria et al., 2013). Further, problematic substance use affects the broader university and neighboring communities, contributing to higher rates of sleep and study disruption, property damage, noise complaints, and verbal, physical and sexual violence (Wechsler and Nelson, 2008).

Effectively addressing substance use requires a coordinated approach across the university and its academic and administrative units that views mental and behavioral health as the foundation for student success. College is one of the few times in a person's life where a single integrated setting encompasses all primary activities, both career-related and social, as well as services related to health, safety, and well-being. This represents a tremendous opportunity to address health and wellness among a segment of the population entering a high-risk developmental phase. Further, by nature of their teaching and research missions, colleges represent an ideal forum from which to develop best-practices for addressing health and wellness. Since many universities possess on their campuses researchers with expertise in relevant areas of study, it is striking that there has been so little systematic research that integrates basic science and intervention research into a unified university approach. There is a need to develop and evaluate programs to improve prevention, identification, and treatment of substance use and mental health problems on college campuses and to translate these findings into policy and practice (Hunt and Eisenerg, 2010).

There is tremendous variability in practices across different campuses, with many using approaches that are sub-optimal (Winters et al., 2011). A survey of colleges examining implementation of the 2002 NIAAA college drinking task force recommendations (http://www.collegedrinkingprevention.gov/) found that the primary approach colleges use to address student alcohol use is education, *a strategy that research has demonstrated is ineffective on its own* (Nelson et al., 2010). Further, only half of surveyed colleges offered empirically supported intervention programs for students who were problem drinkers. Clearly, there is a huge gap between the research basis and translation into best-practices on college campuses.

"Spit for Science": A Translational Initiative Focused on College Behavioral Health

At our university, we have considerable research expertise in the area of substance use and mental health. In the fall of 2011, we launched a university-wide research project focused on substance use and mental health. The scientific goal of the project is in-line with other on-going projects of faculty in this area: to understand how genetic and environmental factors impact the development of substance use and mental health problems. However, Spit for Science is unique in that we partnered from the beginning with campus administration and wellness practitioners to create a university-wide initiative aimed at benefitting our students and our local community.

Methods

All incoming first-time freshmen age 18 or older are invited to complete an on-line survey at the beginning of their fall semester. The survey contains questions about personality and behavior, as well as family, friends, and experiences growing up, and takes approximately 15–30 min to complete. Students receive $10 and a free "Spit for Science" t-shirt for completing the survey. They also have the opportunity to provide a saliva sample for the DNA component (hence the "spit" in Spit for Science), for which they receive another $10. Students have the option of participating in the survey portion of the project and not the DNA component. We do considerable educational outreach about the DNA component and how health outcomes, including substance use and mental health, are a product of our environments and our genetic predispositions. Nearly 70% of eligible freshmen have participated in the project each year, with ∼97% also choosing to participate in the DNA component. The sample is representative of the overall university population in terms of gender and racial/ethnic breakdown. After 4 years of enrolling incoming freshmen, we have over 9,600 students participating in the project. Students are invited to complete a follow-up survey every spring thereafter. Accordingly, the project allows us to understand patterns of substance use and mental health among our students when they start at the university, and the risk and protective factors that impact behavior across their college years (and beyond). For the first cohort, 80% of eligible participants completed the freshman follow-up survey, 59% completed the sophomore follow-up, 53% the junior follow-up, and 45% have completed the senior follow-up (on-going). Students are reminded of spring follow-up surveys via e-mail, mailed information, advertisements around the university and on campus busses, student "recruiters" passing out flyers and manning information tables, dorm visits and educational events. We are currently implementing a number of additional initiatives intended to bolster retention, including feeding back study results to students and enhancing our use of social media to provide a more interactive on-going connection between students and the project.

All participants are enrolled in a registry, allowing faculty across the university to work with the data and focus on areas

of behavioral health specific to their expertise. The registry also allows for selection of individuals with particular characteristics (e.g., phenotypes or genotypes of interest) for more intensive "spin-off" studies. In addition to the primary analyses focused on alcohol use, there are currently additional projects underway examining nicotine use, depression, anxiety, eating disorders, trauma, sleep, physical exercise, parenting, peer and romantic relationships, family history, and more. Much of the success of the project can be attributed to a highly collaborative group of faculty researchers and prevention/intervention staff, as well as university administrators who supported the project and helped us navigate the logistical hurdles involved in launching a cross-campus initiative of this magnitude. Additional details about the data collection and the groundwork that went into launching this university-wide research project can be found in Dick et al. (2014).

Although the project is on-going, basic researchers and prevention practitioners have already started implementing interventions based on survey findings, including a large campus-wide social norms marketing primary prevention intervention. This mass media simultaneously maximizes study participation by raising awareness about the project and acts strategically as an intervention to provide normative results and health information in a motivational style to students. Monthly 1100 posters are placed in bathrooms across campus (examples can be accessed through www.thewell.vcu.edu). Research shows that half the campus are "high-readers" reading over half to all of the publication multiple times.

Initial Results

Rates of substance use in the Spit for Science sample are consistent with other large studies of college-age populations. For example, in Spit for Science 72% of participants report having tried alcohol, compared to 71% in the Monitoring the Future (MTF) post 12th grade assessment (Johnston et al., 2010); the prevalence of marijuana use is 41% in Spit for Science and 44% in MTF (additional details in Dick et al., 2014). In initial analyses we examined patterns of substance use and potential changes in those patterns across the transitional first year of college by conducting a latent class analysis of substance use as reported at the beginning of the fall freshman semester, and midway through the spring semester (Cho et al., under review). At both timepoints, three classes of individuals emerged: (1) polysubstance users, with relatively higher levels of use of alcohol, tobacco, cannabis, and other illicit drugs (2) alcohol, tobacco, cannabis users; and (3) low substance users, with overall low levels of use, with alcohol use being the most common. Interestingly, very few participants transitioned in their substance use class across the first year of college. This suggests that college students largely maintain the patterns of substance use *with which they enter college*. This finding is consistent with a previous study that reported pre-college heavy drinking to be the strongest predictor of heavy drinking in the first semester (Sher and Rutledge, 2007). These studies suggest that high-risk users have previously established substance use patterns prior to college, a finding that has important implications for early intervention efforts: *we know who is most at risk from the time they step onto our campus*. This underscores the importance of implementing effective, empirically supported prevention efforts that go beyond "one-size-fits-all."

Integrating Developmental Epidemiology with Prevention/Intervention

The current "gold standard" for reducing risky alcohol use among college students is the use of brief motivational feedback interventions (Larimer and Cronce, 2002; Lee et al., 2010), which have demonstrated efficacy delivered via in-person and web-based platforms (Chiauzzi et al., 2005; Carey et al., 2009; Hustad et al., 2010). These programs generally combine elements of cognitive-behavioral skill training, and personalized feedback in a motivational interviewing style. They provide students with information about how their drinking compares to campus norms. They also help students see possible consequences associated with excessive alcohol use such as impact on academic performance and career goals, and they empower students to undertake new strategies to monitor their drinking, set limits, and reduce risk. They have been adopted for both universal prevention programming intended for all college students, and targeted programming for mandated students (Barnett et al., 2004; Bosari and Carey, 2005; White et al., 2006; Hustad et al., 2010).

What is striking about this literature is that there is little integration with the body of research on pathways of risk for alcohol problems. Individuals use and abuse alcohol for different reasons (Heinz et al., 2003), and the development of alcohol-related problems is often discussed within the context of multiple pathways. In a study we conducted on the association between early childhood temperamental factors and adolescent alcohol use using data from >12,000 individuals followed from birth, we found two distinct temperamental/behavioral patterns evident before age 5 that predicted mid-adolescent alcohol use: (1) children who were rated as having consistent emotional and conduct problems and (2) children who were rated as consistently sociable both had elevated rates of alcohol use in adolescence (Dick et al., 2013). An externalizing pathway, characterized by behavioral undercontrol, sensation-seeking, impulsivity, and antisocial behavior, has been robustly associated with alcohol problems (Zucker, 2008), and there is more modest evidence for a risk pathway characterized by internalizing symptomatology (Zucker, 2008; Hussong et al., 2011). Further, individuals with alcoholism also show considerable heterogeneity, with a common distinction being alcoholics who have antisocial/externalizing traits and alcoholics who have anxious/depressive comorbid features (Cloninger et al., 1981; Babor et al., 1992). These literatures all indicate that individuals who misuse alcohol are a heterogeneous group. Yet our current prevention/intervention strategies often apply a one-size-fits-all strategy that focuses almost entirely on alcohol use and not on the various factors that (differentially) affect risk.

Recently, a literature has begun to emerge that focuses on prevention programming tailored to individual risk profiles. Conrod et al. (2013) developed a school-based alcohol prevention program that targets personality risk profiles: anxiety sensitivity, hopelessness, impulsivity, and sensation-seeking, and shows robust effects on reducing adolescent drinking behavior (Conrod et al., 2013; O'Leary-Barret et al., 2013). Schuckit et al. (2009)

developed a tailored intervention focused on low level of response to alcohol, a known biological risk factor reflecting a need for larger amounts of alcohol to experience effects, that has been robustly associated with higher alcohol intake and increased risk for the development of alcohol-related problems (Schuckit et al., 2009). Level of response can be assessed using a brief set of self-report questions that ask an individual to report on the number of drinks it took them to experience various effects of alcohol (slurred speech, stumbling, etc.) when they first began drinking. In a pilot study of college freshmen, individuals who reported a low level of response to alcohol and who were assigned to a prevention program structured around how a low physiological response affects heavy drinking, showed greater decreases in alcohol use as compared to individuals with a low level of response who were assigned to a standard prevention program that covered the same information, but not in the context of a low level of response framework. Individuals who did not have a low level of response to alcohol did better in the standard prevention program (Schuckit et al., 2012). We assessed level of response in the Spit for Science survey and used these data to invite a subset of students to participate in an intervention study designed to replicate the Schuckit et al. (2012) finding. We too found modest support for individuals with a low level of response showing decreased alcohol use in the level of response prevention program compared to the non-tailored program, particularly for high risk drinking practices such as maximum number of drinks in a day (Savage et al., unpublished). Interestingly, we found that the individuals with a low level of response (i.e., those most at risk) showed greater decreases in alcohol use to both prevention programs compared to individuals with a high level of response. These findings are consistent with results from another line of prevention work by Brody et al. (2009) in which children who were characterized as at risk based on their genetic profiles showed the greatest benefit from prevention programming aimed at reducing adolescent alcohol use (Brody et al., 2009). Though risk was characterized in different ways across these studies (physiological response versus measured genotypic risk), and different prevention programs were implemented with different populations, both studies found that those at greatest risk benefited most from prevention programming.

We believe that there is great potential to integrate these literatures to develop more targeted prevention and intervention programming for college students that focuses on individual risk factors. Although brief motivational feedback interventions for college student substance use have demonstrated efficacy, the effects associated with these programs are modest (Walters and Neighbors, 2005; Rooke et al., 2010) and risky alcohol use remains widespread on college campuses (Timberlake et al., 2007; Martinez et al., 2008; Wechsler and Nelson, 2008; Kilmer and Geisner, 2013). Personalized feedback is thought to be one of the critical elements contributing to the effectiveness of extant college prevention programs (Walters and Neighbors, 2005). Integrating findings on risk factors associated with alcohol use would make it possible to provide feedback based on more comprehensive risk profiles that extend beyond current patterns of alcohol use. We are currently working on using technology-based platforms to provide individual feedback across multiple dimensions (e.g., level of response, personality, externalizing and internalizing characteristics) in order to test whether enhanced personalized feedback improves prevention/intervention outcomes. The ability to use technology to personalize feedback also obviates the need to group individuals into subtypes (e.g., high/low responders, impulsive, anxious, etc.) as each individual can have their own personalized risk profile. The emerging literature on the enhanced effectiveness of tailored prevention programming suggests this may be a fruitful way forward.

Where Does Genetics Fit In?

As part of the Spit for Science project we collect DNA. We are clear with our students that the DNA will be used for basic research purposes only, to identify genes that are involved in why some people are more likely to develop problems associated with substance use and mental health than others, and to understand how the environment can moderate risk for those who are genetically predisposed. We are explicit that students will not receive feedback about their risk. The science simply is not at a point where that information is useful. This is illustrated by analyses conducted by a graduate student in my (DD) lab who completed a Ph.D. in genetics and genetic counseling in which she evaluated the predictive ability of known genes associated with alcohol dependence and found that at our current level of knowledge they predict no better than chance (Yan et al., 2013). Family history remained the most robust predictor. Polygenic risk scores combining information across the genome currently predict only ∼1–3% of the variance in alcohol-related outcomes (Salvatore et al., 2014). However, as our gene finding efforts advance, the percent of variance explained by known genetic risk factors is likely to grow, as evidenced in other areas where the amount of variance explained by polygenic risk scores has become non-trivial (e. g., 60% for type- I diabetes; 10% for height; Visscher et al., 2012). Identifying these risk genes has required huge samples (180,000 individuals for height! Lango Allen et al., 2010), and efforts to grow large-scale collaborations are underway for substance dependence. However, genetics will always be just part of the puzzle, with substance use disorders having a heritability in the range of 50–70% (Verhulst et al., 2015). Further, it remains unclear how individuals would use personalized genetic information. The FDA's current ban on direct to consumer genetic testing (U.S. Food and Drug Administration, 2013), and the considerable controversy that surrounded the University of California Berkley's provision of genetic results to its students as an academic exercise to stimulate discussion about personalized genetic feedback (Gruber, n.d.), underscore the uncertainty surrounding how personalized genetic information may be integrated into efforts aimed at prevention/intervention and improving human health.

However, these challenges are not insurmountable, and there are ways that genetically informative information can be useful in the interim, even before we have identified genes and have a clearer sense of how to use this information. We know that there are no genes "for" substance use or mental health outcomes anyway. Rather, genetic factors impact distal clinical outcomes through intermediary traits and pathways. For example, in the area of substance use, genetic factors that impact risk for the development of substance use problems likely act through intermediary

mechanisms such as personality and physiological response to alcohol. Twin studies and molecular genetic studies have demonstrated that genetic influences that impact adult alcohol use outcomes can manifest as conduct problems earlier in development, and also impact other indices of behavioral disinhibition, such as sensation-seeking and novelty seeking (Young et al., 2000; Krueger et al., 2002; Dick et al., 2009; Aliev et al., 2015). Accordingly, we can use these more proximal traits that are part of the pathway of risk influenced by the underlying predisposition for personalized feedback, which will allow us to further study how the provision of individual risk information influences the effectiveness of prevention/intervention programming. We think there is great potential for basic researchers to work with prevention/intervention practitioners to develop interactive feedback programming that provides students with more comprehensive information about their profile of risk (potentially including biologically and psychosocially influenced factors), in order to give them insight into factors that affect their substance use and mental health.

Conclusion

University settings provide great potential for translational efforts that bring together basic scientists, prevention/intervention practitioners, and university administrators. Substance use and behavioral health concerns on college campuses are areas of high impact for the university community where these translational efforts hold great promise. The effectiveness of new, personalized prevention programming suggests that integrating etiological information into prevention and intervention efforts may provide new and innovative ways to address challenging, common problems among young adults. Basic scientists can provide information about the pathways of risk involved in behavioral health challenges, while benefitting from collaborative discussions with practitioners that can inform the research. By teaming up with researchers, practitioners can benefit from the knowledge of local research expertise, and enhance their ability to evaluate program effectiveness. Administrators who have access to data showing the connection between substance use and student success outcomes on their campus may have a stronger impetus to fund both the research and intervention programs focused on substance use and mental health at the university. In the end, more collaborative translational research interventions hold great promise for all involved.

Author Contributions

Both authors contributed equally to the creation of this work and approve this version to be published.

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Higher levels of protective parenting are associated with better young adult health: exploration of mediation through epigenetic influences on pro-inflammatory processes

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The current investigation was designed to examine the association of parenting during late childhood and early adolescence, a time of rapid physical development, with biological propensity for inflammation. Based on life course theory, it was hypothesized that parenting during this period of rapid growth and development would be associated with biological outcomes and self-reported health assessed in young adulthood. It was expected that association of parenting with health would be mediated either by effects on methylation of a key inflammatory factor, Tumor necrosis factor (TNF), or else by association with a pro-inflammatory shift in the distribution of mononuclear blood cells. Supporting expectations, in a sample of 398 African American youth residing in rural Georgia, followed from age 11 to age 19, parenting at ages 11–13 was associated with youth reports of better health at age 19. We found that parenting was associated with changes in *TNF* methylation as well as with changes in cell-type composition. However, whereas methylation of *TNF* was a significant mediator of the association of parenting with young adult health, variation in mononuclear white blood cell types was not a significant mediator of the association of parenting with young adult health. The current research suggests the potential value of examining the health-related effects of parenting in late childhood and early adolescence. Further examination of protection against pro-inflammatory tendencies conferred by parenting appears warranted.

Keywords: epigenetic, methylation, african american, parenting, CpG, TNF, SES

Introduction

Recent theorizing has suggested that feeling sick and acting sick are adaptive elements in the response to a variety of ailments (cf. Hart, 1988; Dantzer and Kelley, 2007), and that upregulated inflammatory pathways may play an important signaling role that facilitates the perception of being sick and organizes illness-related behavior. To the extent that inflammatory mechanisms play a central role in regulating perceptions of health and illness, self-reported health potentially provides a useful window on inflammatory mechanisms. Given the importance ascribed to pro-inflammatory processes in

Abbreviations: CpG, regions of DNA in which a cytosine nucleotide occurs next to a guanine nucleotide separated by only one phosphate; TNF, Tumor necrosis factor; SES, socioeconomic status; DMR, Differentially methylated Region

long-term health outcomes and particularly long-term cardiovascular health (Hansson, 2005), better understanding factors contributing to pro-inflammatory tendencies has important implications for models of healthy aging. In particular, a range of social circumstances influence inflammatory processes and health, with negative social interactions resulting in greater inflammation and positive interaction leading to less inflammation (Dickerson et al., 2009; Chiang et al., 2012).

The effect of family influences during youth and early adolescence on later young adult inflammatory processes are anticipated by predictive adaptive response (PAR) models (Gluckman et al., 2005; Rickard and Lummaa, 2007), which note that if earlier family circumstances signal increased probability of future injury and/or pathogen exposure, it is potentially adaptive to prepare a developing young person to have greater inflammatory response potential (cf. Cole et al., 2011). From this perspective, social adversity and perceived threat in childhood should upregulate the innate immune system, a system that provides immediate defense against infection, and enhance pro-inflammatory response tendencies as a way to prepare for potential impending tissue damage and infection. PAR models are also consistent with broader life history theory (Charnov, 1993) in suggesting that enhanced pro-inflammatory tendencies in young adulthood may be triggered by adverse social circumstances during childhood even if such adjustments carry with them the cost of longer-term negative health implications (cf. Belsky et al., 1991; Gibbons et al., 2012).

A series of studies have identified a number of facets of parenting that may contribute to a "protective" approach to parenting in difficult circumstances that may reduce perceived threat and adversity for youth (Brody et al., 1994, 2002, 2004; Brody and Flor, 1998; Wills et al., 2000, 2003; Gibbons et al., 2004). These "protective parenting" practices foster self-regulation, academic competence, psychological adjustment, and avoidance of substance related problems (Wills et al., 2000; Brody et al., 2002; Gibbons et al., 2004) among African American youth. The cluster of protective parenting practices includes both high levels of nurturant, involved, and supportive parenting interactions as well as low levels of harsh or inconsistent parenting. Such parenting interactions lead to a lower frequency of destructive arguments and a high level of youth perceived support from parents. The constellation of "protective parenting" practices conveys security, stability, and safety at home, creating a context that would be expected to protect against pro-inflammatory remodeling. Whereas as parenting relationships characterized by lack of support and harsh parent-child interaction would be expected to have a pro-inflammatory effect.

Better understanding of the way family processes during childhood contribute to or protect against inflammation is of particular importance for African Americans who have greater prevalence, earlier onset and more complications from inflammation related diseases, including cardiovascular disease (Hozawa et al., 2007), and type 2 diabetes. African Americans also have a 30% greater chance of dying of cardiovascular disease relative to whites (Office of Minority Health, 2012), a twofold greater risk of type-II diabetes and increased likelihood of being affected by complications of type 2 diabetes, including heart disease, blindness, amputations, stroke and death (Konen et al., 1999). Increasing evidence suggests these conditions and complications are driven by inflammatory processes). African Americans also tend to show higher levels of inflammatory markers (Geronimus et al., 2006; Chyu and Upchurch, 2011; Paalani et al., 2011) than do whites. Thus, better understanding factors that protect against inflammatory processes may be particularly useful in identifying risk and protective factors for African American health in young adulthood.

Life history theory (Charnov, 1993) provides a broad framework for hypothesizing two mechanisms that may relate parenting to inflammation, particularly parenting during periods that are characterized by rapid developmental change such as late childhood and early adolescence. Less supportive and more harsh parenting should accelerate speed of development (Belsky et al., 1991; Figueredo et al., 2005) and lead to shifts in the innate immune system to better respond to a harsh interpersonal environment. Recent theorizing within this tradition suggests that these shifts may be manifested in changes in the relative frequency of particular cell types in blood (Irwin and Cole, 2011) as well as in the epigenetic programming and gene expression of such cells (Miller et al., 2011a). These two different types of effects on early programming of adaptive responses (Gluckman et al., 2005; Rickard and Lummaa, 2007) suggest the need to examine two indirect pathways through enhanced inflammatory potential to health outcomes among young adults. At the same time, epigenetic effects of parenting are plausible given previously observed epigenetic effects from parenting to later outcomes through effects on methylation in animal models (Champagne et al., 2008; Trollope et al., 2011), and a growing body of research reporting epigenetic effects of early childhood experience for humans (e.g., Essex et al., 2013; Beach et al., 2014).

If verified, the influence of protective parenting in later childhood and early adolescence on young adult health may be particularly important because parenting practices are a potentially modifiable point of intervention that could be used to ameliorate health disparities (Brody et al., 2012). Prior work has shown that family support and problem-solving skills delivered during later childhood and early adolescence can help protect youth from adverse physiological stress reactions (Chen et al., 2011; Brody et al., 2014) whereas parental maltreatment or other adverse events in childhood contribute to vulnerability to chronic diseases later in life (Repetti et al., 2002; Shonkoff et al., 2009).

Although the way in which protective parenting during childhood and adolescence can be turned into biological changes with health consequences for young adulthood is not yet fully understood (cf. Hertzman, 1999), one way that upregulated propensity for inflammation could occur is through epigenetic programming of immune cells (Miller et al., 2011a) by changing methylation of specific CpG sites (i.e., regions of DNA in which a cytosine nucleotide occurs next to a guanine nucleotide separated by only one phosphate), thereby influencing access to elements controlling rate of gene transcription. Because methylation of CpG islands associated with the first exon is particularly predictive of gene expression (e.g., Plume et al., 2012), characterizing individual differences in methylation of inflammation-related genes in the region of the first exon may be particularly informative. A second pathway from parenting to effects on inflammatory tendencies could result from changes in the relative frequency

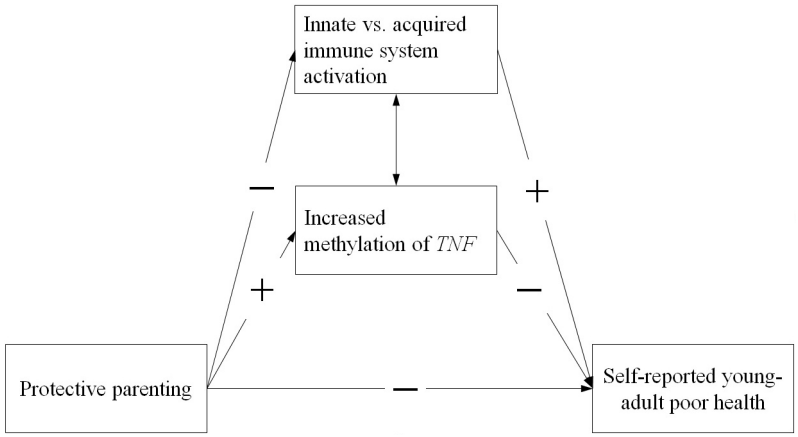

FIGURE 1 | The conceptual model showing two potential biological, indirect pathways from protective parenting to self-reported young adult poor health. (1) A positive efffect (+) on level of methylation of TNF, and (2) a negative effect (−) on activation of monocytes reflecting greater innate immune system activation, with each associated in turn with young adult health.

of particular immune cell types linked to inflammation (Irwin and Cole, 2011). Individuals with more cells from the innate immune system would be prepared to mount a more robust innate immune system response, providing a greater pro-inflammatory context for reactions to the environment. More specifically, epigenetic programming of cells would allow them to show more pronounced inflammatory responses when exposed to challenge (Miller et al., 2011a), an effect that has been observed in primate models (Cole et al., 2012), as well as in humans (Irwin and Cole, 2011). Likewise, a changed distribution of inflammation related cells, such as an increase in the proportion of innate immune system cells such as monocytes (aka CD14 cells) relative to T or B cells (aka CD4, CD8, and CD19) could indicate a shift toward a pro-inflammatory response pattern.

The two different types of potential effects on early programming of pro-inflammatory responses (Gluckman et al., 2005; Rickard and Lummaa, 2007) suggest the need to examine two indirect pathways in models examining potential biological mechanisms of influence from parenting to later health outcomes. With regard to epigenetic change of inflammation related genes, we focus on methylation of *TNF* due to the central role of TNF-α in inflammatory processes. All cells involved in inflammation have receptors for TNF-α and are activated by it to facilitate further inflammation. This positive feedback quickly amplifies the acute phase inflammatory response, making *TNF* an attractive focus of empirical attention. With regard to cell-type variation, we examine variation in concentration of cell-types in lymphocyte pellets to identify variation that may reflect relatively greater responsiveness of the innate vs. the acquired immune system. The resulting general model is portrayed in **Figure 1**. To the extent that the significant indirect effects (IE) suggested in the figure are identified, they focus theoretical attention on biological mechanisms potentially conferring long-lasting effects of protective parenting on health. Accordingly, we examine the hypothesis that protective parenting during childhood and early adolescence will be associated with self-reported health in young adulthood, leading to a negative (-) association and that inflammatory mechanisms in the form of differences in *TNF* methylation and cell-type variation will account for some or all of this association. In the process of examining three main hypotheses, we also examine three measurement hypotheses:

Main hypotheses

1. (a) Protective parenting will be associated with epigenetic regulation of inflammation via methylation of *TNF* resulting in a positive (+) association as well as with relative activation of innate vs. acquired immune responses via shifts in the relative proportion of different white blood cell types of different white blood cell types leading to a negative (-) association.
   (b) Protective parenting will be associated with perceived health, indicating its implications for predicting young adult health.
2. (a) The methylation index created to capture variation in level of methylation of exon 1 of *TNF*, and a cell-type index capturing a shift toward pro-inflammatory processes, will demonstrate associations with level of protective parenting as well as with perceived health, allowing direct comparison of their role in mediating the impact of protective parenting on young adult health.
   (b) Level of methyation of *TNF* will be associated with cell-type variation such that hypothesized pro-inflammatory patterns will tend to be mutually reinforcing.
3. As portrayed in **Figure 1**, both methylation of *TNF* and shifts in cell-type variation will mediate the effect of protective parenting on perceived health in young adulthood and be associated with significant IE from parenting to health.

Measurement Hypotheses:

1. Batch and Chip effects will be identified in the full set of CpGs assessed and confirmed using technical replicates, allowing them to be controlled.

2. Cell type variation will be characterized utilizing patterns of DMRs, allowing characterization of shifts in relative proportions of cells associated with innate vs. acquired immune system response.
3. Degree of methylation of CpG residues on the first exon of *TNF* will be intercorrelated, indicating coordination of methylation across the first exon, and indicating that an index of methylation of the first exon for *TNF* results in a reliable construct.

Materials and Methods

Participants

A total of 398 primary caregivers (PCs) and target youth residing in rural Georgia, were selected randomly from a larger sample of youth participating in an ongoing longitudinal study, and provided data yearly between youth ages of 11 and 19. Participants resided in nine rural counties in Georgia. In these counties, families live in small towns and communities in which poverty rates are among the highest in the U.S. and unemployment rates are above the national average (Dalaker, 2001). Recruitment and data collection procedures were developed with input from focus groups of rural African American community members (Kumpfer et al., 2002; Murry and Brody, 2004), and community liaisons were used to aid in the recruitment and retention of participants. Community liaisons were African American community members selected on the basis of their social contacts and standing in the community. The community liaisons sent a letter to the families and followed up on the letter with phone calls to the PCs. To enhance rapport and cultural understanding, African American students and community members served as home visitors to collect data at all visits.

Self-report questionnaires were administered to mothers and target children in an interview format. Each interview was conducted privately, with no other family members present or able to overhear the conversation. Assessments of parenting were provided by African American PCs. 96.2% of PCs were female, with 89.21% being the biological mother. Other females in the role of PC were Aunt (1.05%), Grandmother 4.21%, step-mother (0.26%). When males were the PCs they were the biological father (3.42%). Foster father (0.26%), or grandfather (0.79%). Mean age of PCs was 46.122 (SD = 7.540), 15.70% had less than a 12th grade education, and 65.50% had a job. At the first assessment, PCs in the sample worked an average of 39.9 h per week, and 42.3% lived below federal poverty standards, with a majority living below 150% of the poverty threshold, and median monthly family income was $1,710. Median monthly family income was $1,648 at the age of 19. In this and other regards they are representative of the Georgia counties in which they reside (Boatright and Bachtel, 2003).

Youth provided blood for epigenetic assessments as well as reports of the caregiver's parenting and their own physical health. Target youth mean age was 11.7 years at the first assessment and 19.2 years at the time of epigenetic assessment based on a blood draw. Of the 398 targets, 0.8% were married at the time of the blood draw, and another 1.5% were separated, divorced, or widowed. Of the young adults whose outcomes are the focus of the investigation, 45.4% are males and 54.6% of females. Approximately one-quarter (24%) had less than a 12th grade education, and only 7.3% had a full-time job.

The current sample has been the focus of prior research described in Beach et al. (in press), and Beach et al. (2014).

Procedure

A standardized assessment protocol lasting 2 h, on average, was used at each wave of data collection. All data were collected in participants' residences using two African American field researchers who met with each family to allow separate and simultaneous data collection from the PC and the target youth. All interviews were conducted so that no other family members were present or able to overhear the conversation. PCs consented to their own and the youths' participation in the study, and the youths under 18 assented to their own participation and then consented when they participated as adults. All procedures were approved by the University of Georgia Institutional Review Board.

Measures

Parenting

Protective parenting processes related to support, communication, and monitoring as well as adverse practices such as harsh parenting were assessed across five scales using target youth reports as well as parent reports. Three scales were common to youths and PCs with wording changes as appropriate. The Interaction Behavior Questionnaire (IBQ; Prinz et al., 1979), Nurturant-Involved Parenting Scale (Conger et al., 1994), and the Harsh/Inconsistent Parenting Scale (Brody et al., 2001) were completed by both youths and a PC. Youths also completed a revised version of the four-item Social Support for Emotional Reasons subscale from the COPE scale to assess levels of parental support (Carver et al., 1989). PCs also completed the Destructive Arguing Inventory to assess styles of conflict and conflict resolution within parent-child relationship (Kurdek, 1994). Not including harsh parenting, Cronbach's alphas ranged from 0.73 to 0.84 for caregivers and from 0.76 to 0.85 for youth. Harsh parenting displayed lower alphas than other measures (0.54 to 0.60 for caregivers; 0.53 to 0.59 for youths).

Each scale was standardized and then averaged across the first three waves of assessment (i.e., ages 11–13). We reversed negatively valenced parenting measures to ensure that for all measures, higher scores indicated more protective parenting and fewer negative practices. All the parenting measures were summed to form the overall measure of protective parenting.

Young Adult Health

Youths reported their general health in young adulthood (age 19) using the General Health Perceptions subscale from the RAND Short-Form Health Survey (Hays et al., 1993) shortly after the blood draw to assess methylation. This five-item subscale includes a single-item rating of overall health ranging from 1 (*excellent*) to 5 (*poor*) and four items assessing youths' ratings of their current health status ranging from 1 (*definitely false*) to 5 (*definitely true*); e.g., "I am as healthy as anybody I know"; "I seem to get sick a little easier than other people." In keeping with standard scoring,

responses 1 through 5 were recoded to values of 100, 75, 50, 25, 0. Positive items were reversed scored so that higher scores indicated more health problems and poorer general health. After reverse scoring, all items were averaged to yield a General Health Problems score with a range of 0–100 ($\alpha = 0.76$).

BMI and Diet

Body mass index (BMI) was calculated at ages 18 and 19 as weight in kilograms divided by the square of height in meters. In the current study, mean BMI was 28.33 (SD = 8.13), with 54.6% of the participants classified as overweight (BMI $\geq$ 25). Healthy diet was assessed at ages 18 and 19 using two items that asked about frequency of fruit and vegetable consumption during the previous 7 days. The relationship between the two items was significant $r = 0.483$ ($p < 0.001$). Responses ranged from 1 (none) to 5 (twice a day or more) and were averaged to form the healthy diet variable.

Methylation

Certified phlebotomists drew four tubes of blood (30 ml) from each participant. Tubes were shipped the same day to a laboratory for preparation. After receipt, the blood tubes were inspected to ensure anticoagulation and aliquots of blood were diluted 1:1 with phosphate buffered saline pH 8.0. Mononuclear cell pellets were separated from the diluted blood specimen by centrifugation with ficoll (400 g, 30 min) and the mononuclear cell layer was removed from the tube using a transfer pipette, resuspended in a phosphate buffered saline solution, and briefly centrifuged again. The resulting cell pellet was resuspended in a 10% DMSO/RPMI solution and frozen at −80°C until use. A typical DNA yield for each pellet was between 10 and 15 mg.

The Illumina (San Diego, CA, USA) HumanMethylation450 Beadchip was used to assess genome-wide DNA methylation. Participants were randomly assigned to 12 sample "slides/chips" with groups of eight slides being bisulfite converted in a single batch, resulting in five "batches/plates." A replicated sample of DNA was included in each plate to aid in assessment of batch variation and to ensure correct handling of specimens. The replicate sample was examined for average correlation of beta values between plates and was found to be greater than 0.99. Prior to normalization, methylation data were filtered based on these criteria: (1) samples containing 1% of CpG sites with detection p-value > 0.05 were removed, (2) sites were removed if a beadcount of < 3 was present in 5% of samples and (3) sites with a detection p-value of > 0.05 in 1% of samples were removed. More than 99.76% of the 485,577 probes yielded statistically reliable data.

Quantile normalization of methylation data

Recent demonstrations (e.g. Pidsley et al., 2013) have shown that quantile normalization methods as well as separate normalization of Type I and Type II assays in the Illumina array produce marked improvement in detection of relationships by correcting distributional problems inherent in the manufacturers default method for calculating β (i.e., $\beta = M/(M + U + 100)$ where M and U are methylated and unmethylated signal intensities, respectively. Accordingly, for the current investigation, the methylated and unmethylated intensities obtained using the Illumina HumanMethylation450 BeadChip were quantile normalized using the wateRmelon (2013) R package (Team, 2012; Schalkwyk et al., 2013). The "*dasen*" function recommended by Pidsley et al. (2013) was used. This method equalizes the backgrounds of Type I and Type II probes prior to normalization, and includes between-array normalization of Type I and Type II probes separately but does not perform dye bias correction.

Identifying and correcting for chip and batch effects

As demonstrated by Sun et al. (2011), quantile normalization typically reduces, but may not eliminate, batch and chip effects. Accordingly, after preprocessing to normalize the beta values, all samples were examined for batch and chip effects. The distribution of quantile normalized average β values for all samples in each chip and batch were contrasted with all others using a box and density plot to indicate both the mean and confidence intervals around the mean in each case. The results of this examination are provided in the preliminary stage of the results section. Any plate or chip effects can then be controlled in all subsequent analyses. Because all plates contained a technical replicate, it was also possible to confirm the batch/plate effects via direct examination of the replicated sample.

Assessing proportion of cell types in mixed cell populations

Ficoll purified peripheral blood mononuclear cell pellets of the sort used in the current investigation are comprised of several different cell types (e.g., CD4, CD8, CD14, CD19, CD56) (Reinius et al., 2012). To account for individual differences in cell types, such as that potentially produced by a shift toward pro-inflammatory innate immune system cell types, a regression calibration approach similar to that developed by Houseman et al. (2012) was performed. However, whereas Houseman et al. (2012) used an approach based on the Illumina HumanMethylation 27K BeadChip, we utilized an alternative approach based on Illumina HumanMethylation 450K BeadChip data. Highly informative CpG sites were identified by contrasting methylation profiles for purified cells (CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells, and CD56+ Natural Killer cells) using data contributed by (Reinius et al., 2012; GEO database under accession number GSE35069) and analyses were performed using regression in MethLAB, Version 1.5 (Kilaru et al., 2012). The 100 sites best differentiating to the five cell types of interest were retained for further analysis in SPSS version 22 (IBM Corporation Released, 2013). A locus determined to be on the X chromosome was dropped from subsequent analyses. Then, we performed a principal components analysis (PCA) on the remaining 99 loci using the current data set to identify principle component factors that would characterize dimensions of individual variability in cell-type in the most parsimonious manner for the current data set.

Results

Results are presented in six steps, beginning with the three steps reflecting preliminary, measurement-related analyses and then the three steps related to specific hypotheses.

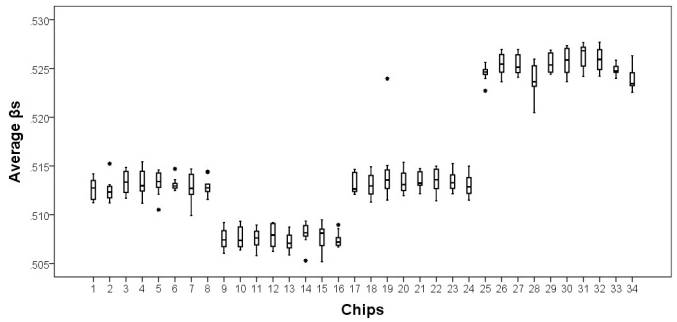

FIGURE 2 | Average methylation values across all CpG sites by chip. Chips 1–8 chips on Plate 1; chips 9–16 chips on Plate 2; chips 17–24 on Plate 3; chips 25–32 on Plate 4; chips 33–34 on Plate 5. There are plate effects but not chip effects, with Plates 4 and 5 having higher average methylation values and Plate 2 having a lower average Beta than do Plates 1 and 3. *indicates the presence of an outlier from the chip.

Measurement-related Analyses

Characterizing Cell Type Variation

As described above, we extracted the 99 loci best differentiating cell-types in the Reinius et al. (2012) data set, and then subjected these to a principle component factor analysis. The scree plot had an elbow after the first three factors, with the first three factors accounting for 31.06%; 14.26%; and 6.64% of the common variance respectively. To determine which, if any of these factors might reflect a shift toward greater innate vs acquired immune response, we used a simultaneous regression to predict factor scores from cell types using the original data set from Reinius et al. (2012). Greater scores on the first principle component (PC1) were inversely associated with CD56 (Natural Killer Cells) and positively associated with CD4 (T helper cells) and CD19 (B or T helper cells), suggesting a relationship to both innate and acquired immune system activity. The second principle component (PC2) was associated negatively with CD19 (B or T cells) and positively with CD8 (T suppressor cells), albeit non-significantly in all cases, a pattern not clearly reflective of a shift toward innate or acquired responses. PC3 was positively and robustly associated with CD14 (monocytes) and negatively associated with CD19 (acquired B or T cells), suggesting a shift toward greater responsiveness of the innate immune system. All principle component scores were also examined at the zero order level to identify associations with parenting and young adult health to identify which could potentially mediate effects of protective parenting.

Characterizing Batch and Chip Effects

To examine the distribution of quantile normalized average βs indicating level of methylation, all methylation values for each sample were contrasted with all others using a box and density plot to indicate both the mean and confidence intervals around the mean for each chip and batch. As can be seen in **Figure 2**, confidence intervals for chips within batches overlap suggesting no need for correction of chip effects. However, confidence intervals for batches did not overlap, suggesting there is a need for correction of batch/plate effects.

The pattern of means observed for the technical replicate reproduced the pattern observed across all quantile normalized average βs, confirming the need to correct for batch/plate effects. Accordingly, batch/plate was used as a categorical covariate in all analyses.

TNF Methylation

Eight CpG sites were identified as being associated with the first exon of *TNF*. Greater methylation of this region for cells expressing TNF should result in a reduction in gene expression and so, ultimately, a reduction in TNF-α. The intercorrelation of the eight CpG values were examined (*r*s ranging from 0.736 to 0.942; all *p*s < 0.00001) and all individual CpGs were significantly correlated with parenting (*r*s ranging from 0.094 to 0.172) and with young adult health (*r*s ranging from −0.080 to −0.143). A factor analysis of the eight CpGs identified a single factor with all loadings above 0.85. Accordingly, to index overall methylation of the first exon of *TNF*, βs for CpGs on the first exon were mean-centered and standardized prior to creating an average score with a Cronbach alpha of 0.98.

Model-related Analyses

Hypothesis 1a

As can be seen in **Table 1**, protective parenting was associated with the *TNF*-index ($r = 0.150$, $p = 0.003$). However, of the three principle components comprising variation in cell types, protective parenting was associated significantly only with PC3 ($r = -0.166$, $p = 0.001$).

Hypothesis 1b

Protective parenting was also associated significantly with young adults' reports of health in young adulthood ($r = -0.108$, $p = 0.031$).

Hypothesis 2a

As can be seen in **Table 1**, the methylation index for *TNF* was associated with young adult health ($r = -0.123$, $p = 0.014$), as well as with cell-type variation (PC1 $r = 0.662$, $p = 0.000$; PC2 $r = 0.334$, $p = 0.000$; PC3 $r = -0.350$, $p = 0.000$), setting the stage for tests of mediation. However, of the factors

TABLE 1 | Correlation matrix for the major study variables ($N = 398$).

	1	2	3	4	5	6	7	8	9	10
1. Parenting	–									
2. Self-reported health	−0.108*	–								
3. *TNF*-index	0.150**	−0.123*	–							
4. Male	0.007	−0.095†	0.066	–						
5. Age	0.037	−0.124*	0.104*	0.014	–					
6. BMI	−0.005	0.098†	−0.034	−0.128*	0.055	–				
7. Diet	0.011	−0.079	−0.035	−0.003	0.042	0.036	–			
8. Factor 1	0.075	−0.041	0.662**	−0.149**	0.103*	0.006	−0.008	–		
9. Factor 2	0.035	−0.094†	0.334**	0.128*	0.093†	−0.008	−0.085	0.003	–	
10. Factor 3	−0.166**	0.039	−0.350**	−0.218**	−0.019	0.021	0.018	−0.005	−0.011	–
Mean	−0.131	26.043	0.001	0.452	20.464	0.548	2.835	0.084	0.017	0.031
SD	4.375	18.721	0.938	–	0.607	0.498	0.854	30.618	14.263	6.499

$^{**}p \leq 0.01$; $^{*}p \leq 0.05$; $^{\dagger}p < 0.10$ (two-tailed tests). Factors 1–3 are the three principle components reflecting cell-type variation in the current data.

capturing cell-type variation, only factor 3 was associated both with parenting ($r = -0.166$, $p = 0.001$). Accordingly, the *TNF*-index and Factor 3 were examined as potential alternative pro-inflammatory mediators of the effect of parenting on young adult health.

Hypothesis 2b

The *TNF*-index was also associated in the expected direction with PC3 ($r = -0.350$, $p = 0.000$), indicating that less methylation of *TNF* (pro-inflammatory) was associated with relatively greater presence of monocytes (pro-inflammatory).

Hypothesis 3

Using the *TNF*-index and PC3 to represent alternative potential pro-inflammatory pathways linking protective parenting to health, we examined whether the effects of protective parenting on self-reported health were mediated by methylation and/or cell type variation. We used the function (MODEL INDIRECT) in Mplus version 7.2 (Muthén and Muthén, 1998–2012) and obtained bootstrap confidence intervals for the effect of the independent variables (parenting and SES risk exposure) on the outcome variable (young adult self-reported health) through the mediator (methylation index) using 1000 replicates to assess the bias-corrected 95% confidence intervals for the IE (Hayes, 2009). This approach estimates direct and IE simultaneously, does not assume a standard normal distribution when calculating the *p*-value for the IE, and repeatedly samples the data to estimate the IE.

In the final model, shown in **Figure 3**, which drops non-significant pathways from control variables, parenting is associated with both youth reported health and with *TNF*-methylation. We found that parenting was associated with the *TNF*-methylation index score as well as with Factor 3 reflecting cell-type variation in a multivariate context (See **Figure 3**), controlling for batch/plate effects. As can be seen in **Table 2**, the impact of protective parenting on self-reported health in young adulthood was partially, but not fully, mediated by impact on the *TNF*-methylation index, accounting for 11.19% of the variance in young adult health, with a significant IE of parenting on young adult health of $IE = -0.047$, 95% CI $= (-0.127, -0.003)$ and a significant unstandardized direct effect of $B = -0.42$, 95% CI $= (-0.790, -0.048)$. Factor 3, however, id not mediate the effects of parenting on young adult health, $IE = 0.022$, 95% CI $= (-0.047, 0.109)$.

Discussion

The examination of the way that protective parenting during late childhood and early adolescence influences health and potentially remodels biological systems through epigenetic change is just beginning. To the best of our knowledge, this is the first study to contrast potential effects of protective parenting during late childhood and early adolescence on pro-inflammatory process through epigenetic regulation of gene expression vs. increased presence of pro-inflammatory innate immune system cell-types (e.g., monocytes). Consistent with study hypotheses, we found that protective parenting practices, assessed longitudinally, were associated with effects on pro-inflammatory processes both through effects on methylation of *TNF*, and through effects on proportion of immune cells that were monocytes. Accordingly, parenting at ages 11–13 was negatively associated with both pro-inflammatory epigenetic patterns and pro-inflammatory cell-type variation assessed at age 19. In addition, these effects were not spurious associations due to age, gender, or batch (i.e., measurement) effects. Because we used a longitudinal research design to test hypotheses regarding the effects of parenting on health, we identified protective parenting as a predictor of pro-inflammatory processes and so strengthened its claim to play a causal role in young adult health. These findings are consistent with the proposition that poor health and health disparities during young adulthood may be ameliorated, in part, by processes correlated with protective parenting.

The current results suggest that better understanding the way in which mononuclear white blood cell signaling processes are altered, net of effects on individual differences in cell type composition, is promising as a mechanism by which protective parenting influences young adult pro-inflammatory tendencies among African American youth. The current study builds on past results by suggesting that there may be effects of protective parenting in late childhood and early adolescence that parallel or expand upon

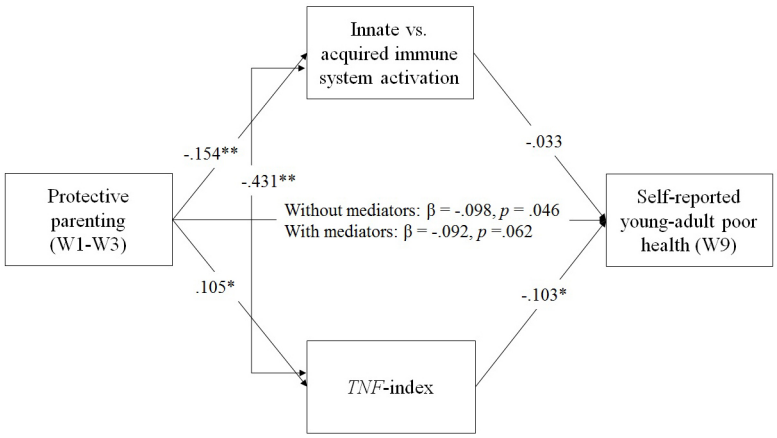

FIGURE 3 | Mediated effect of protective parenting on youth reported health in young adulthood, controlling for sex, age, and batch effects, freeing non-significant pathways from control variables.

Chi-square = 4.124, *df* = 3, $p = 0.248$; CFI = 0.996; RMSEA = 0.031. Values are standardized parameter values. Sex, age, BMI, diet, and batch/plate are controlled. $N = 398$. $^{*}p < 0.05$, $^{**}p < 0.01$, two-tailed.

TABLE 2 | Significance of the indirect effects on health through factor 3 and TNF ($N = 398$).

Paths	Total effect	Indirect effect	The portion of the variance for mediator
Protective Parenting (W1–W3) → Innate vs. acquired immune system activation → Self-reported young-adult poor health (W9)	−0.420* (−0.790, −0.048)	0.022 (−0.047, 0.109)	−%
Protective Parenting (W1–W3) → TNF-index activation → Self-reported young-adult poor health (W9)	−0.420* (−0.790, −0.048)	−0.047* (−0.127, −0.003)	11.190%

*The values presented are standardized parameters. Bootstrapping with 1,000 replications. *$p \leq 0.05$, (two-tailed test).*

those observed for early childhood life stress on subsequent biological and genomic functioning (e.g., Miller et al., 2011b; Essex et al., 2013). The current results suggest that protective parenting measured during late childhood and early adolescence may also exert an influence on genomic functioning and health in young adults, and contributes to promising work on multiple fronts suggesting that various epigenetic mechanisms may be related to, and help account for, long-term effects of protective parenting on health.

At the same time, our findings provided only partial support for the dual pathway model outlined in **Figure 1**. First, although it was possible to characterize the relative proportion of mononuclear white blood cell types in the blood samples provided using the techniques developed by Accomando et al. (2014) and Houseman et al. (2012) to characterize cell type variation, and we found that protective parenting was associated with the relative proportion of monocytes in the mononuclear white blood cell sample, that proportion was not associated with youth reports of their health in young adulthood. Accordingly, there was no evidence of mediation of parenting effects on young adult health through cell-type variation in the current sample. In addition, even the significant mediational pathway through *TNF* methylation did not account for the majority of health related effects of protective parenting during late childhood and early adolescence. Only 11.19% of the variance in young adult health attributable to protective parenting was accounted for by *TNF* methylation, suggesting that there are other pathways to young adult health that require explication.

In addition, limitations of the present study design that preclude strong causal conclusions should be noted. Because we did not assess early childhood parenting, we do not know if the observed effects for protective parenting during late childhood and early adolescence might be accounted for by even more potent effects in early childhood. Future work with measures of both early and later parenting would be helpful in resolving this concern. In addition, there were no repeated measurements of either level of *TNF* methylation or variability in cell-type composition. As a consequence, it is not possible to address the issue of change in inflammatory mechanisms and whether protective parenting in late childhood was associated with this change. It would be useful if future replications with younger rural African American children could examine the interplay of environmental challenges and parenting occurring at multiple stages of development to better characterize key developmental stages at which protective parenting exerts its greatest effects. Likewise it would be useful to examine whether protective parenting has different effects on inflammatory processes at different ages. Accordingly, replication of the current investigation with repeated measurement of methylation as well as repeated measurement of protective parenting would be useful. Finally, because we did not measure gene expression, the current results await replication and extension using methods that can

clarify whether the observed effects are associated with changes in gene expression that would indicate up or down regulation of *TNF*. Additional limitations include absence of objective medical records, lack of information about possible in utero exposures, lack of specific somatometric assessments of truncal fat, lack of specific dietary assessment of PUFA consumption, lack of explication of potential co-caregiver effects, and absence of genotyping of TNF polymorphisms. However, we controlled for BMI and general aspects of healthy diet and found no significant association with TNF methylation, finding that these did not affect the observed relationships. Likewise, self-reported health just prior to the blood draw indicated that the young adults who comprised the current sample considered themselves relatively healthy on average at the time of the blood draw and they were not yet at an age where a general population sample of this sort would be anticipated to have accumulated large numbers of physician documented health problems. Finally, to the extent that specific genetic polymorphisms influence TNF methylation, they would not be anticipated to affect the observed results unless they were correlated with parenting as well as TNF methylation.

There is also room for more fine-grained examination of parenting. Protective parenting is comprised of both increased positive and decreased negative forms of parent-child interaction. Accordingly, more fine-grained examination of different facets of parenting could be useful in determining whether some facets of parenting are particularly consequential with regard to particular outcomes. More fine grained analyses could be useful, for example, in developing implications for more targeted intervention to enhance health. There is also a particular need for replication and extension of our finding that greater protective parenting was associated with a significant shift in cell-type composition in the direction of relatively fewer CD14 monocytes (a marker of innate immunity), suggesting potential long-term impact on chronic inflammation (Irwin and Cole, 2011). Although controlling the effect of cell type did not reduce the direct effect of parenting on health or result in a significant IE of protective parenting on young-adult health, this effect does illustrate the potential for protective parenting to influence future health-relevant processes indirectly by changing cell type. To the extent that such changes in cell-type composition enhance reactivity to future life stress, exacerbate the negative effects of health behaviors, accelerate other aging related processes, or change signaling processes that influence other behavior patterns, they may be quite consequential for long-term health outcomes even though that is not apparent in the current investigation. Accordingly, future examination of effects related to impact on cell-type variation is warranted.

Despite limitations and the need for future replication, the current results provide a useful demonstration of the impact of protective parenting during pre-adolescence on *TNF* methylation and youths' long-term health outcomes. Jointly, the results suggest the value of continued investigation of epigenetic changes related to protective parenting and its potential for impact on later health outcomes.

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