# Problem Behavior in Adolescence

## Testing the influence of stress reactivity, autoantibodies and methylation

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# Problem Behavior in Adolescence: Testing the influence of stress reactivity, autoantibodies and methylation

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# Introduction

## Background

Externalizing and internalizing problems often develop during adolescence, and are associated with an increased risk for mental health disorders in adulthood. Externalizing problems can manifest itself in antisocial behavior or problematic substance use [1-3], and internalizing problems can develop into depression or anxiety disorders [4]. It is therefore important to understand the physiological and psychological mechanisms behind the development of externalizing and internalizing problems during adolescence, as this might open up new possibilities for early identification of adolescents at risk, and help in the development of prevention programs and possibly new treatments.

One of the factors associated with both externalizing and internalizing problems is stress, in the broadest sense. Adolescence is a period of quick development and change, both physiologically (e.g. hormonal changes, fast neurological development [5,6]), and psychologically (e.g. changes in relationships between adolescents and their parents versus their peers [7,8]). These developmental changes increase the amount of stressors experienced during adolescence, and might even make adolescents more sensitive to the negative effects of stress. This is reflected in an increased risk of developing externalizing or internalizing disorders in young adulthood in those who experienced lots of stressful life events during adolescence [9-13]. Underlying reasons for this association could be a heightened emotional response to stress associated with the differential development of subcortical emotional systems and cortical control systems [14], or changes in coping skills during adolescence [10,15]. Another mechanism which could be responsible for this link is a dysregulation of the body´s stress response mechanism (stress reactivity), which normally helps the body to react appropriately to a stressor by initiating the fight and flight response [16]. If this mechanism is dysregulated, the body will not be able to deal appropriately with stressors any longer, and subsequently the risk of developing externalizing and internalizing problems will increase. Indeed, a dysregulated stress reactivity is often observed in persons suffering from externalizing [17,18] or internalizing problems [19]. However, research into the relationship between a dysregulated stress reactivity and externalizing or internalizing problems has yielded inconsistent results. Some studies have shown associations between blunted stress reactivity and an increased risk for developing externalizing and internalizing problems [20-24], while others did not find any relationship [25], or even a higher stress reactivity in relation to externalizing and internalizing problems [26-30]. Thus, more research is needed to clarify the directionality and mechanism of the relationship between stress reactivity and externalizing and internalizing problems, and into identifying new factors that could play a role in explaining this complex relationship.

This thesis focuses on a novel and exciting new area of research in this field, which is whether natural autoantibodies against neuropeptides influence stress reactivity,

emotions and behavior. It further focuses on the role of stressful life events and stress reactivity in the continuation and escalation of cannabis use and finally investigates the relation between methylation of the catechol-O-methyltransferase (COMT)-gene and substance use.

## Physiological and psychological stress reactivity

One of the most important physiological stress systems, and a focus of this thesis, is the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1). Upon encountering a stressful situation, the hypothalamus secretes corticotropin-releasing hormone (CRH), which in turn triggers the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary [31,32].

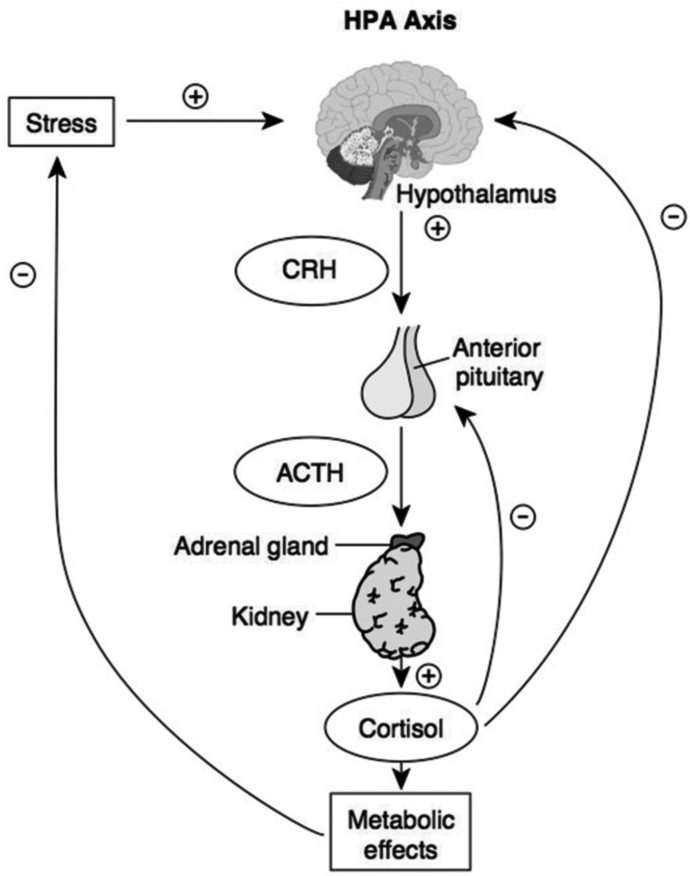


**Figure 1: Schematic presentation of the HPA axis.** Abbreviations: HPA, hypothalamic-pituitary-adrenal; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone. From: S Hiller-Sturmhöfel, A Bartke. The endocrine system: an overview. Alcohol Health Res World: 1998, 22(3); 153-64.

ACTH then induces the secretion of cortisol from the adrenal glands, which can be measured in saliva with a 20-minute delay [33]. After activation, a negative feedback mechanism is initiated that deactivates HPA axis reactivity [34]. This mechanism of activation and subsequent deactivation is well regulated and necessary for our body to generate an appropriate stress response. Another characteristic of the HPA axis is its diurnal rhythm. Shortly after awakening there is a steep rise in cortisol, which probably prepares the body for the activities of the upcoming day [35]. The HPA axis is a very delicate system and it is involved in regulating a range of bodily functions such as energy metabolism and immune system functioning [36,37]. Interestingly, it has been found that HPA axis activity changes during the transition from childhood to adolescence: Both basal cortisol levels [38-40] and HPA axis reactivity in response to social stress have been shown to increase with age and sexual maturation [41,42]. Animal models support the notion of a change in HPA axis reactivity during adolescence, for example, adolescent rats show an extended HPA axis responses after a stressor and an impaired glucocorticoid-dependent negative feedback compared to adult rats [43]. The increase in HPA axis reactivity observed during adolescence has been suggested to contribute to a heightened risk of developing psychopathology, e.g., depression, during this time [42].

Besides physiological stress reactivity, one also experiences psychological stress, i.e., the degree to which a stressor is perceived as stressful [44]. Psychological stress reactivity can be measured as perceived arousal before, during and after a stress test [45]. Perceived arousal was found to be related to HPA axis reactivity in response to social stress and to anxiety symptoms [46,47]. Thus, not only physiological stress reactivity, but also perceived arousal in response to a stressor might be related to externalizing and internalizing problems in adolescence.

## Relation between stress reactivity and externalizing and internalizing problems in adolescence

A dysregulation of the HPA axis is often observed in persons suffering from externalizing or internalizing disorders [17-19,28]. However, studies have been inconsistent as to whether hyper- or hypo-reactivity of the HPA axis is related to externalizing and internalizing problems. No explanation is available for the discrepancies in the data, which demonstrates the need to investigate new biological mechanisms that could influence the relationship between externalizing and internalizing problems and HPA axis reactivity.

While some studies have shown that externalizing problems in adolescence were related to a hypo-reactivity of the HPA axis [20-23,48,49], other studies have shown that externalizing problems were related to a hyper-reactivity of the HPA axis [26,27]. Regarding substance use in particular, it is known that acute and chronic substance use increases HPA axis reactivity [50-52]. On the other hand, a hypo-reactivity of the HPA axis in response to stress has been observed in cannabis users [53], and was also related to early initiation of cannabis use [54]. One explanation for

these discrepancies might be that biological factors, e.g., genetic polymorphisms, or environmental factors, e.g., stress, moderate the relationship between HPA axis reactivity and externalizing problems. For example, a recent study has shown that stress moderates the relationship between HPA axis reactivity and externalizing behavior [55]. More studies like this are important to determine the exact nature of such moderation effects. Another question that needs further attention is that of why adolescents often experiment with substance use, but only few develop problematic substance use. Problematic substance use can lead to addiction and is associated with difficulties at school, bad health, family conflicts and financial problems [56]. Thus, there is a strong need to investigate the reasons for why some adolescents develop problematic substance use while others do not. The underlying reason for this is not known, but stressful life events and stress reactivity might play a role in making someone more vulnerable to continuing substance use or even becoming a problematic user [57].

Similar to externalizing problems, the development of internalizing problems such as depression or anxiety has been related to dysregulations in the HPA axis [28], with inconsistent study results. One explanation for this discrepancy could be a time-dependent relationship between HPA axis reactivity and internalizing behaviors. It has been suggested that early on during the development of internalizing or externalizing problems a hyper-reactivity of HPA axis is observed, which over time, when symptoms turn chronic, turns into hypo-reactivity [19,58].

The underlying mechanisms by which HPA axis reactivity might be involved in the development of externalizing or internalizing problems are still not fully understood, raising the question of whether new biological mechanisms exist that could influence this relationship. Two possible mechanisms explored in this thesis are the involvement of autoantibodies against neuropeptides and that of gene methylation in regulating HPA axis functioning and externalizing and internalizing problems.

## Autoantibodies as regulators of HPA axis reactivity and behavior

A new exciting area of research is that on natural autoantibodies against neuropeptides, and their possible influence on HPA axis reactivity, emotions and behavior. Antibodies against ACTH, for example, were first identified in patients with eating disorder [59] and related to both externalizing and internalizing behaviors in specific patient groups, for example patients with anorexia [60], Sjörgen´s syndrome (an autoimmune disorder) [61], and inmates with conduct disorder [62]. In addition, animal models have revealed a link between autoantibodies against neuropeptides such as alpha-MSH and ghrelin and appetite, anxiety and depressive symptoms [63,64]. Autoantibodies against neuropeptides belong to the so-called natural autoantibodies, which are ubiquitous, and might be involved in the regulation of physiological functions in the body [65,66], for example the HPA axis. By influencing and possibly dysregulating HPA axis reactivity they could be involved in the development of externalizing and internalizing problems. Thus far, relationships between neuropeptide autoantibodies,

HPA axis reactivity and externalizing or internalizing problems have not been investigated in the general population. Moreover, the exact working mechanism by which autoantibodies against neuropeptides could influence HPA axis reactivity, externalizing and internalizing behaviors, is still unknown. It has been suggested that, upon binding their antigen (the substance that stimulates antibody production, in this case for example ACTH), they transport it to the receptors and thereby enhance its activity [63]. This mechanism has been shown for ghrelin antibodies [64]. For ACTH or alpha-MSH antibodies, the working mechanism has not yet been proven. Besides transportation as a mechanism, another possibility is that the antibodies bind and neutralize their respective antigen, thereby reducing its activity [63]. It has further been suggested that the affinity of such autoantibodies might determine whether they neutralize their antigen or act as transporters [63]. The origin of neuropeptide autoantibodies is unknown, but a likely reason for their formation is molecular mimicry. The molecular mimicry theory states that autoantibodies against self-peptides could be formed due to peptides from pathogens that have similar peptide sequences [67]. Molecular mimicry plays a role in the formation of α-MSH autoantibodies in mice [68], but the concept has not been tested in humans or for other neuropeptides.

## The COMT gene and risk for substance use

An interesting new area of research that should be considered when investigating the development of problem behaviour, e.g., substance use, is that of epigenetics. The dopaminergic reward system plays a role in substance use [69], but no studies so far have investigated the combined effects of genetics and epigenetics on this system, and whether this could be related to substance use in adolescents. It is known that a genetic polymorphisms in the Catechol-O-methyltransferase (COMT) gene, which encodes for an enzyme that degrades dopamine, can influence vulnerability to substance use. The functional Val$^{108/158}$Met single nucleotide polymorphism (SNP) in the *COMT* gene has been associated with altered COMT activity and with substance use [70-73]. Most studies link the Val/Val polymorphism, which results in a more active COMT, to an increased risk for substance use, but findings are inconsistent [74]. Therefore, it is important to test other possible mechanisms of how COMT activity could be related to substance use. One possible mechanism is the regulation of COMT activity via methylation. Methylation of DNA is a form of epigenetic modification. By adding a methyl group to a certain stretch of DNA, often a promoter region, gene expression can be suppressed, resulting in lower levels of the corresponding protein. *COMT* gene methylation has been found to decrease COMT levels [75,76]. Thereby, it could also affect dopamine levels in the brain and hence, substance use. Thus, not only SNPs in the *COMT* gene, but also *COMT* gene methylation could influence dopamine levels and thereby the risk for substance use, but this has not been tested so far.

# 1 Aims and research questions

The general aim of this thesis is to investigate the connections between physiological and psychological stress reactivity and externalizing and internalizing problems in adolescence. Special attention is paid to the identification of new biological mechanisms that could be involved in the development of externalizing and internalizing problems. In particular, the following research questions are addressed:

a. Are autoantibodies against ACTH related to antisocial behavior and stress induced cortisol responses in adolescence?

b. Are autoantibodies against melanocortin peptides (ACTH and α-MSH) related to internalizing problems in adolescence?

c. Are autoantibodies against ghrelin associated with internalizing problems and stress-induced cortisol responses in adolescence?

d. Is there a reciprocal relationship between stressful life events and cannabis use during adolescence?

e. Are physiological and psychological stress reactivity in adolescence related to problematic cannabis use in young adulthood?

f. Is methylation of the COMT gene related to substance use in adolescence?

## References


1. Brook JS, Zhang C, Brook DW (2011) Developmental trajectories of marijuana use from adolescence to adulthood: personal predictors. Arch Pediatr Adolesc Med 165: 55-60.
2. Korhonen T, van Leeuwen AP, Reijneveld SA, Ormel J, Verhulst FC, et al. (2010) Externalizing behavior problems and cigarette smoking as predictors of cannabis use: the TRAILS Study. J Am Acad Child Adolesc Psychiatry 49: 61-69.
3. Miettunen J, Murray GK, Jones PB, Maki P, Ebeling H, et al. (2013) Longitudinal associations between childhood and adulthood externalizing and internalizing psychopathology and adolescent substance use. Psychol Med: 1-12.
4. Pine DS, Cohen P, Gurley D, Brook J, Ma Y (1998) The risk for early-adulthood anxiety and depressive disorders in adolescents with anxiety and depressive disorders. Arch Gen Psychiatry 55: 56-64.
5. Dahl RE (2004) Adolescent brain development: a period of vulnerabilities and opportunities. Keynote address. Ann N Y Acad Sci 1021: 1-22.
6. Lupien SJ, McEwen BS, Gunnar MR, Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci 10: 434-445.
7. Cameron JL (2004) Interrelationships between hormones, behavior, and affect during adolescence: complex relationships exist between reproductive hormones, stress-related hormones, and the activity of neural systems that regulate behavioral affect. Comments on part III. Ann N Y Acad Sci 1021: 134-142.
8. Steinberg L, Morris AS (2001) Adolescent development. Annu Rev Psychol 52: 83-110.
9. Ge X, Conger RD, Elder GH, Jr. (2001) Pubertal transition, stressful life events, and the emergence of gender differences in adolescent depressive symptoms. Dev Psychol 37: 404-417.
10. Seiffge-Krenke I (2000) Causal links between stressful events, coping style, and adolescent symptomatology. J Adolesc 23: 675-691.
11. Turner RJ, Lloyd DA (2004) Stress burden and the lifetime incidence of psychiatric disorder in young adults: racial and ethnic contrasts. Arch Gen Psychiatry 61: 481-488.
12. Kim KJ, Conger RD, Elder GH, Jr., Lorenz FO (2003) Reciprocal influences between stressful life events and adolescent internalizing and externalizing problems. Child Dev 74: 127-143.
13. Low NCP, Dugas E, O'Loughlin E, Rodriguez D, Contreras G, et al. (2012) Common stressful life events and difficulties are associated with mental health symptoms and substance use in young adolescents. Bmc Psychiatry 12.
14. Casey BJ, Jones RM, Levita L, Libby V, Pattwell SS, et al. (2010) The storm and stress of adolescence: insights from human imaging and mouse genetics. Dev Psychobiol 52: 225-235.
15. Skinner EA, Zimmer-Gembeck MJ (2007) The development of coping. Annu Rev Psychol 58: 119-144.
16. Goldstein DS (2010) Adrenal responses to stress. Cell Mol Neurobiol 30: 1433-1440.
17. Alink LR, van Ijzendoorn MH, Bakermans-Kranenburg MJ, Mesman J, Juffer F, et al. (2008) Cortisol and externalizing behavior in children and adolescents: mixed meta-analytic evidence for the inverse relation of basal cortisol and cortisol reactivity with externalizing behavior. Dev Psychobiol 50: 427-450.
18. van Goozen SH, Fairchild G, Snoek H, Harold GT (2007) The evidence for a neurobiological model of childhood antisocial behavior. Psychol Bull 133: 149-182.

19. Ruttle PL, Shirtcliff EA, Serbin LA, Fisher DB, Stack DM, et al. (2011) Disentangling psychobiological mechanisms underlying internalizing and externalizing behaviors in youth: longitudinal and concurrent associations with cortisol. Horm Behav 59: 123-132.

20. Haltigan JD, Roisman GI, Susman EJ, Barnett-Walker K, Monahan KC (2011) Elevated trajectories of externalizing problems are associated with lower awakening cortisol levels in midadolescence. Dev Psychol 47: 472-478.

21. McBurnett K, Lahey BB, Rathouz PJ, Loeber R (2000) Low salivary cortisol and persistent aggression in boys referred for disruptive behavior. Arch Gen Psychiatry 57: 38-43.

22. Poustka L, Maras A, Hohm E, Fellinger J, Holtmann M, et al. (2010) Negative association between plasma cortisol levels and aggression in a high-risk community sample of adolescents. J Neural Transm 117: 621-627.

23. Shoal GD, Giancola PR, Kirillova GP (2003) Salivary cortisol, personality, and aggressive behavior in adolescent boys: a 5-year longitudinal study. J Am Acad Child Adolesc Psychiatry 42: 1101-1107.

24. Petrowski K, Wintermann GB, Schaarschmidt M, Bornstein SR, Kirschbaum C (2013) Blunted salivary and plasma cortisol response in patients with panic disorder under psychosocial stress. Int J Psychophysiol 88: 35-39.

25. Klimes-Dougan B, Hastings PD, Granger DA, Usher BA, Zahn-Waxler C (2001) Adrenocortical activity in at-risk and normally developing adolescents: individual differences in salivary cortisol basal levels, diurnal variation, and responses to social challenges. Dev Psychopathol 13: 695-719.

26. McBurnett K, Raine A, Stouthamer-Loeber M, Loeber R, Kumar AM, et al. (2005) Mood and hormone responses to psychological challenge in adolescent males with conduct problems. Biol Psychiatry 57: 1109-1116.

27. van Bokhoven I, Van Goozen SH, van Engeland H, Schaal B, Arseneault L, et al. (2005) Salivary cortisol and aggression in a population-based longitudinal study of adolescent males. J Neural Transm 112: 1083-1096.

28. Pariante CM, Lightman SL (2008) The HPA axis in major depression: classical theories and new developments. Trends Neurosci 31: 464-468.

29. Pruessner M, Hellhammer DH, Pruessner JC, Lupien SJ (2003) Self-reported depressive symptoms and stress levels in healthy young men: associations with the cortisol response to awakening. Psychosom Med 65: 92-99.

30. Rao U, Hammen C, Ortiz LR, Chen LA, Poland RE (2008) Effects of early and recent adverse experiences on adrenal response to psychosocial stress in depressed adolescents. Biol Psychiatry 64: 521-526.

31. Tsigos C, Chrousos GP (2002) Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53: 865-871.

32. Hiller-Sturmhofel S, Bartke A (1998) The endocrine system: an overview. Alcohol Health Res World 22: 153-164.

33. Kirschbaum C, Hellhammer D (1992) Assessment of Hormones and Drugs in Saliva in Biobehavioral Research. Seattle: Hogrefe & Huber 19-32.

34. Jacobson L, Sapolsky R (1991) The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr Rev 12: 118-134.

35. Fries E, Dettenborn L, Kirschbaum C (2009) The cortisol awakening response (CAR): facts and future directions. Int J Psychophysiol 72: 67-73.

36. Reiche EM, Nunes SO, Morimoto HK (2004) Stress, depression, the immune system, and cancer. Lancet Oncol 5: 617-625.

37. Brunner EJ, Hemingway H, Walker BR, Page M, Clarke P, et al. (2002) Adrenocortical, autonomic, and inflammatory causes of the metabolic syndrome: nested case-control study. Circulation 106: 2659-2665.

38. Legro RS, Lin HM, Demers LM, Lloyd T (2003) Urinary free cortisol increases in adolescent caucasian females during perimenarche. J Clin Endocrinol Metab 88: 215-219.

39. Adam EK (2006) Transactions among adolescent trait and state emotion and diurnal and momentary cortisol activity in naturalistic settings. Psychoneuroendocrinology 31: 664-679.

40. Walker EF, Walder DJ, Reynolds F (2001) Developmental changes in cortisol secretion in normal and at-risk youth. Dev Psychopathol 13: 721-732.

41. Stroud LR, Foster E, Papandonatos GD, Handwerger K, Granger DA, et al. (2009) Stress response and the adolescent transition: performance versus peer rejection stressors. Dev Psychopathol 21: 47-68.

42. Gunnar MR, Wewerka S, Frenn K, Long JD, Griggs C (2009) Developmental changes in hypothalamus-pituitary-adrenal activity over the transition to adolescence: normative changes and associations with puberty. Dev Psychopathol 21: 69-85.

43. Romeo RD (2013) The Teenage Brain: The Stress Response and the Adolescent Brain. Curr Dir Psychol Sci 22: 140-145.

44. Cohen S, Kamarck T, Mermelstein R (1983) A global measure of perceived stress. J Health Soc Behav 24: 385-396.

45. Bradley MM, Lang PJ (1994) Measuring emotion: the Self-Assessment Manikin and the Semantic Differential. J Behav Ther Exp Psychiatry 25: 49-59.

46. Oldehinkel AJ, Ormel J, Bosch NM, Bouma EM, Van Roon AM, et al. (2011) Stressed out? Associations between perceived and physiological stress responses in adolescents: the TRAILS study. Psychophysiology 48: 441-452.

47. Dieleman GC, van der Ende J, Verhulst FC, Huizink AC (2010) Perceived and physiological arousal during a stress task: can they differentiate between anxiety and depression? Psychoneuroendocrinology 35: 1223-1234.

48. Shirtcliff EA, Granger DA, Booth A, Johnson D (2005) Low salivary cortisol levels and externalizing behavior problems in youth. Dev Psychopathol 17: 167-184.

49. Snoek H, Van Goozen SH, Matthys W, Buitelaar JK, van Engeland H (2004) Stress responsivity in children with externalizing behavior disorders. Dev Psychopathol 16: 389-406.

50. Monteleone P, Di Filippo C, Fabrazzo M, Milano W, Martiadis V, et al. (2014) Flattened cortisol awakening response in chronic patients with schizophrenia onset after cannabis exposure. Psychiatry Res 215: 263-267.

51. Murphy LL, Munoz RM, Adrian BA, Villanua MA (1998) Function of cannabinoid receptors in the neuroendocrine regulation of hormone secretion. Neurobiol Dis 5: 432-446.

52. Steptoe A, Ussher M (2006) Smoking, cortisol and nicotine. Int J Psychophysiol 59: 228-235.

53. van Leeuwen AP, Creemers HE, Greaves-Lord K, Verhulst FC, Ormel J, et al. (2011) Hypothalamic-pituitary-adrenal axis reactivity to social stress and adolescent cannabis use: the TRAILS study. Addiction 106: 1484-1492.

54. Huizink AC, Ferdinand RF, Ormel J, Verhulst FC (2006) Hypothalamic-pituitary-adrenal axis activity and early onset of cannabis use. Addiction 101: 1581-1588.

55. Jaffee SR, McFarquhar T, Stevens S, Ouellet-Morin I, Melhuish E, et al. (2015) Interactive effects of early and recent exposure to stressful contexts on cortisol reactivity in middle childhood. J Child Psychol Psychiatry 56: 138-146.

56. Copeland J, Swift W (2009) Cannabis use disorder: epidemiology and management. Int Rev Psychiatry 21: 96-103.

57. Gruber AJ, Pope HG, Jr. (2002) Marijuana use among adolescents. Pediatr Clin North Am 49: 389-413.

58. Booij SH, Bouma EM, de Jonge P, Ormel J, Oldehinkel AJ (2013) Chronicity of depressive problems and the cortisol response to psychosocial stress in adolescents: the TRAILS study. Psychoneuroendocrinology 38: 659-666.

59. Fetissov SO, Hallman J, Oreland L, Af Klinteberg B, Grenback E, et al. (2002) Autoantibodies against alpha -MSH, ACTH, and LHRH in anorexia and bulimia nervosa patients. Proc Natl Acad Sci U S A 99: 17155-17160.

60. Fetissov SO, Harro J, Jaanisk M, Jarv A, Podar I, et al. (2005) Autoantibodies against neuropeptides are associated with psychological traits in eating disorders. Proc Natl Acad Sci U S A 102: 14865-14870.

61. Karaiskos D, Mavragani CP, Sinno MH, Dechelotte P, Zintzaras E, et al. (2010) Psychopathological and personality features in primary Sjogren's syndrome--associations with autoantibodies to neuropeptides. Rheumatology (Oxford) 49: 1762-1769.

62. Fetissov SO, Hallman J, Nilsson I, Lefvert AK, Oreland L, et al. (2006) Aggressive behavior linked to corticotropin-reactive autoantibodies. Biol Psychiatry 60: 799-802.

63. Sinno MH, Do Rego JC, Coeffier M, Bole-Feysot C, Ducrotte P, et al. (2009) Regulation of feeding and anxiety by alpha-MSH reactive autoantibodies. Psychoneuroendocrinology 34: 140-149.

64. Takagi K, Legrand R, Asakawa A, Amitani H, Francois M, et al. (2013) Anti-ghrelin immunoglobulins modulate ghrelin stability and its orexigenic effect in obese mice and humans. Nat Commun 4: 2685.

65. Lutz HU, Binder CJ, Kaveri S (2009) Naturally occurring auto-antibodies in homeostasis and disease. Trends Immunol 30: 43-51.

66. Merbl Y, Zucker-Toledano M, Quintana FJ, Cohen IR (2007) Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. J Clin Invest 117: 712-718.

67. Oldstone MB (2005) Molecular mimicry, microbial infection, and autoimmune disease: evolution of the concept. Curr Top Microbiol Immunol 296: 1-17.

68. Tennoune N, Chan P, Breton J, Legrand R, Chabane YN, et al. (2014) Bacterial ClpB heat-shock protein, an antigen-mimetic of the anorexigenic peptide alpha-MSH, at the origin of eating disorders. Transl Psychiatry 4: e458.

69. Robinson TE, Berridge KC (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev 18: 247-291.

70. Beuten J, Payne TJ, Ma JZ, Li MD (2006) Significant association of catechol-O-methyltransferase (COMT) haplotypes with nicotine dependence in male and female smokers of two ethnic populations. Neuropsychopharmacology 31: 675-684.

71. Vandenbergh DJ, Rodriguez LA, Miller IT, Uhl GR, Lachman HM (1997) High-activity catechol-O-methyltransferase allele is more prevalent in polysubstance abusers. Am J Med Genet 74: 439-442.

72. Redden DT, Shields PG, Epstein L, Wileyto EP, Zakharkin SO, et al. (2005) Catechol-O-methyl-transferase functional polymorphism and nicotine dependence: an evaluation of nonreplicated results. Cancer Epidemiol Biomarkers Prev 14: 1384-1389.

73. Lachman HM, Papolos DF, Saito T, Yu YM, Szumlanski CL, et al. (1996) Human catechol-O-methyltransferase pharmacogenetics: description of a functional polymorphism and its potential application to neuropsychiatric disorders. Pharmacogenetics 6: 243-250.

74. Tammimaki AE, Mannisto PT (2010) Are genetic variants of COMT associated with addiction? Pharmacogenet Genomics 20: 717-741.

75. Sasaki M, Kaneuchi M, Sakuragi N, Dahiya R (2003) Multiple promoters of catechol-O-methyltransferase gene are selectively inactivated by CpG hypermethylation in endometrial cancer. Cancer Res 63: 3101-3106.

76. Abdolmaleky HM, Cheng KH, Faraone SV, Wilcox M, Glatt SJ, et al. (2006) Hypomethylation of MB-COMT promoter is a major risk factor for schizophrenia and bipolar disorder. Hum Mol Genet 15: 3132-3145.

# chapter 2

## Corticotropin (ACTH)–reactive immunoglobulins in adolescents in relation to antisocial behavior and stress-induced cortisol response. The TRAILS study.

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## Abstract

Elevated levels of corticotropin (ACTH)-reactive immunoglobulins (ACTH IgG) were found in males with conduct disorder, suggesting their involvement in the biology of antisocial behavior. We first aimed to confirm these findings in a large general population sample of adolescents. Secondly, we studied the association between ACTH IgG levels and hypothalamic-pituitary-adrenal (HPA) axis response to stress.

Free and total ACTH IgG levels were measured in sera of 1230 adolescents (15-18 years). HPA axis activity was determined by measuring salivary cortisol before, during, and after a social stress test. Antisocial behavior was assessed using the Antisocial Behavior Questionnaire. ACTH peptide and IgG affinity kinetics for ACTH were assayed in a subsample of 90 adolescents selected for high or low ACTH IgG levels.

In boys, higher total ACTH IgG levels were associated with higher antisocial behavior scores ($\beta$=1.05, p=0.04), especially at high levels of free ACTH IgG. In girls, antisocial behavior was associated with low free ACTH IgG levels ($\beta$=-0.20, p=0.04). Stress-induced cortisol release was associated with free ACTH IgG in boys ($\beta_{\text{area under the curve}}$=-0.67, p<0.01), and with total ACTH IgG in girls ($\beta_{\text{recovery}}$=0.84, p=0.05). The affinity kinetics assay showed that ACTH IgG association rates were lower in both boys and girls with high ACTH IgG levels.

These data show that ACTH IgG levels are related to antisocial behavior and HPA axis response to stress in adolescents. The mechanisms behind these associations, including different ACTH binding properties of IgG in subjects with antisocial behavior, deserve further attention.

## Introduction



Antisocial behavior often develops during childhood and adolescence and can manifest itself in delinquency. Research into neurobiological mechanisms involved in antisocial behavior has focused on the hypothalamic-pituitary-adrenal (HPA) axis, an important regulator of the physiological stress response. Its dysgregulation has been linked to externalizing problems [1-3]. While some studies have reported associations of aggression and antisocial behavior with low cortisol levels [4-7], others have found no association [8], or higher cortisol levels in adolescents with externalizing behavior problems [9,10]. The inconsistent findings and weak associations between cortisol and antisocial behavior increase the need for investigating other factors of HPA axis functioning that could influence antisocial behavior. Corticotropin (ACTH)-reactive immunoglobulins (IgG) are one such factor to explore.

High levels of ACTH IgG were found in prisoners and males with conduct disorder [11], suggesting their association with antisocial behavior, maybe through interfering with the HPA axis and altering stress-related behavior. It is plausible that ACTH IgG influence cortisol release upon stress by modulating ACTH action. This in turn could result in stress-induced behavior changes like impulsivity and aggression [12]. A similar model has previously been proposed for IgG against a-MSH, an ACTH-derived peptide [13]. According to this model, neuropeptide-reactive IgG may play a role in peptide transportation, protecting peptides from degradation by peptidases. An increase in affinity of such IgG would result in neutralization, decreasing peptide transportation and biological activity [13]. Both unbound ('free') IgG and IgG in immune complexes circulate in the blood. To be able to distinguish between the roles of free and total IgG, it may be informative to look at both pools separately [14,15].

The previous study of ACTH IgG and antisocial behavior included males with severe conduct problems (n=20), prisoners (n=20), and healthy volunteers (n=22) [11]. It was the first study to demonstrate elevated ACTH IgG levels in aggressive and violent subjects compared to healthy controls. In this study, antisocial behavior of the controls was not measured and likely the prisoners were at the extreme end of the antisocial behavior spectrum. The study could not adjust for potential covariates (e.g. age, socioeconomic status). Our study is the first to investigate associations between ACTH IgG and both antisocial behavior and HPA axis response in a large sample of adolescents from the general population, making it possible to adjust for covariates. Most studies on antisocial behavior and low cortisol levels have focused on males [4-6,16], but a similar pattern has been reported in girls [17]. Both HPA axis and the immune system are differentially regulated in males and females [18]. Knowing this, we were interested in exploring sex differences in our study.

Thus, the aim of this study was to validate the finding that ACTH IgG levels are associated with antisocial behavior in a large sample of male adolescents, and to investigate if such associations could also be found in females. Furthermore, we hypothesized that ACTH IgG are associated with HPA axis activity. We tested this

by linking serum ACTH IgG levels to cortisol responses during a social stress test. We further characterized ACTH IgG properties by studying their affinity kinetics in adolescents with high or low ACTH IgG levels.

## Methods

### Participants

The TRacking Adolescents' Individual Lives Survey (TRAILS) is a cohort study that follows young adolescents (10-12 years) into adulthood [19]. The study was approved by the National Dutch Medical Ethics Committee. Informed consent was required for participation, for each assessment separately. Measurements, consisting of validated questionnaires, interviews and biological measures, are taken every two to three years. The first assessment wave ran from March 2001 to July 2002 (T1). During T1, 2230 children were enrolled (response rate 76.0%; de Winter et al 2005), of whom 1816 (81.4%) participated in T3 (September 2005 to December 2007). During T3, 744 adolescents were invited to perform a series of laboratory tasks (experimental session) on top of the usual assessments, of whom 715 (96.1%) agreed to do so. The costly and labor-intensive nature of the laboratory tasks precluded assessing the whole sample. Adolescents with a higher risk of mental health problems had a greater chance of being selected for the experimental session. High risk was defined based on T1 measures of temperament (high frustration and fearfulness, low effortful control), lifetime parental psychopathology, and living in a single-parent family. In total 66.0% of the focus sample had at least one of the above described risk factors; the remaining 34.0% were selected randomly from the low-risk TRAILS participants. The focus sample still represented the whole range of problems seen in a normal population of adolescents. The present study focused on data from T3 (15-18 years). Blood serum was available from 1230 participants (573 boys). Of these participants, 590 (286 boys) took part in the experimental session, including the Groningen Social Stress Test (GSST), a modified version of the Trier Social Stress Test [20].

The average time between the GSST and blood draw was 12.8 weeks. In 80% of all subjects the GSST and blood draw were not more than 18 weeks apart, half of the subjects had the GSST before and half after the blood draw. We would like to point out that IgG have a half-life of 28 days in the blood, therefore we can assume ACTH IgG levels to be a rather stable biological factor.

Adolescents using corticosteroids (n=9) were excluded from the cortisol analyses. Girls using oral contraceptives (OC, n=98) were analyzed separately, since an earlier study of this sample indicated that OC-users have a blunted cortisol response towards the GSST [20]. For ACTH peptide and IgG affinity measurements we selected 45 adolescents (23 boys) with high and 45 (22 boys) with low total ACTH IgG levels. Exclusion criteria were taking OC or corticosteroids.

### The social stress test

The GSST took place in soundproof rooms with blinded windows; it started between 08:00 and 09:30 or between 12:00 and 14:30 and lasted about three hours and 15 minutes. Participants were asked to refrain from smoking and from having coffee, milk, chocolate, and other sugar-containing foods in the two hours before the test. Although free salivary cortisol levels are higher in the morning due to the circadian rhythm of cortisol production, morning and afternoon cortisol responses to social stress were expected [21] and found to be comparable in a previous study of this sample [20]. The GSST was done using a standardized protocol, inspired by the Trier Social Stress Task [22] for the induction of moderate performance-related social stress. Participants were instructed to prepare a six-minute speech about themselves and deliver this speech in front of a video camera. They were told that their videotaped performance would be judged on content of speech as well as on use of voice and posture, and rank-ordered by peers after the experiment. Participants had to speak continuously for six minutes. The test assistant watched the performance critically, without showing empathy or encouragement. After the speech, a three minute rest interlude followed. Then, participants were instructed to subtract 17 repeatedly, starting with 13,278, while the test assistant gave negative feedback. This mental arithmetic task lasted six minutes, followed by three minutes of silence, after which the participants were thoroughly debriefed about the experiment.

## Measures

### Antisocial behavior

Antisocial behavior was assessed using the *Antisocial Behavior Questionnaire* (ASBQ, Cronbach's alpha = .86) [23,24]. The ASBQ rates antisocial and delinquent behaviors in the preceding twelve months. It consists of 25 items (e.g., 'Have you destroyed anything on purpose?', 'Have you used a weapon?', 'Have you been in a physical fight?', 'Have you stolen anything from others?'). Questions can be rated as (0) no, never, (1) once, (2) two or three times, (3) four to six times, (4) seven times or more. Complete data on antisocial behavior and ACTH IgG levels was available for 1195 adolescents (554 boys).

### Cortisol

The HPA axis response during the GSST was assessed by four saliva cortisol samples, considering the normal delay (20-25 minutes) in peak cortisol response to experimental stressors [25]: (1) before the test, representing pre-test cortisol levels, (2) directly after the test, reflecting HPA axis responses during speech, (3) 20 minutes after the test, reflecting levels at the end of the test, and (4) 40 minutes after the end of the GSST, representing post-test cortisol levels. Samples were stored at -20°C until analysis.

Cortisol was assessed from saliva by the Salivette sampling device (Sarstedt, Numbrecht, Germany). Cortisol concentrations were determined in duplicates from

100µl saliva, using an in-house radioimmunoassay, applying a polyclonal rabbit cortisol antibody and 1,2,6,7 $^{3}H$ cortisol (Amersham, Arlington Heights, IL). After incubation at 60°C, the bound and free fractions were separated using active charcoal. The intra-assay coefficient of variation was 8.2% for concentrations of 1.5nM, 4.1% for concentrations of 15nM, and 5.4% for concentrations of 30nM. The inter-assay coefficients of variation were, respectively, 12.6%, 5.6%, and 6.0%. The detection limit was 0.9nM. The amount of cortisol released during the GSST (incremental area under the curve with respect to increase, AUCincr) was calculated over four measures taken during the GSST, using the trapezoid formula described by Janssens et al. [26]. Recovery was calculated by subtracting post-test values from the peak.

## ACTH Ig and peptide measurements

ACTH Ig levels (IgG n=1230, IgM and IgA n=92) were measured in serum using an enzyme-linked immunosorbent assay (ELISA) [27]. Briefly, 96-well plates (Nunc Immunoplate, Rochester, NY) were coated with 2mg/ml ACTH (Bachem AG, Bubendorf, Switzerland) in 0.5M $Na_2CO_3$, 0.5M $NaHCO_3$, pH 9.6, and incubated for 72 hours at 4°C. Plates were washed using phosphate buffered saline (PBS, 0.05% Tween20, pH 7.4). Serum was diluted 1:100 in either physiological buffer (PBS, 0.02% sodium azide, pH 7.4) to measure free ACTH IgG or 3M NaCl, 1.5M glycine (pH 8.9), creating a dissociative condition to measure total ACTH IgG levels. After incubation at 4°C overnight, plates were washed and incubated for 3h with alkaline phosphatase-conjugated secondary antibody (anti-human IgG, IgA or IgM; Jackson ImmunoResearch, West Grove, PA). The reaction was developed for 40min with 150µl p-Nitrophenyl phosphate substrate solution (Sigma, St Louis, MO) at 1mg/ml in 0.1M Tris-HCl, 0.1M NaCl, 5mM $MgCl_2$-$6H_2O$, pH 9.

100µl NaOH (2M) was added to stop the reaction and optical density (OD) was measured at 405nm in an ELISA plate reader. The average difference between duplicates was 5.2%. ACTH peptide concentration was measured in 80 sera according to manufacturers' instructions (ACTH KIT, Phoenix Pharmaceuticals, Burlingame, CA), with an average difference of 4.1% between duplicates. Concentrations of serum total IgG, IgA and IgM were determined in 80 sera by nephelometry.

## IgG purification and affinity kinetics analysis

Peptides were removed from 250µl serum (n=90) using C-18 SEP column (Phoenix Pharmaceuticals, Burlingame, CA, USA) and serum total IgG was purified using the Melon Gel IgG Spin Purification System (ThermoScientific, Rockford, IL), both according to manufacturers' instructions. We used the Biacore 1000 instrument (GE Healthcare, Pscataway, NJ) to measure affinity kinetics. ACTH peptide was diluted at 0.5mg/ml in sodium acetate buffer and coupled covalently on a CM5 sensor chip (GE Healthcare). Serum total IgG was diluted to five concentrations to run a multi-cycle method for affinity kinetics: 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 0.0625mg/ml and 0.03125mg/ml in HBS-EP buffer (GE Healthcare). In each cycle, 60µl were injected

with a flow rate of 30µl/min at 25°C. Association and dissociation rate constants (Ka and Kd) and affinity constants at equilibrium (KD) were calculated by applying a Langmuir 1:1 binding model (BiaEvaluation software).



## Covariates

Current use of OC was assessed before the GSST. Menstrual cycle phase was calculated based on the first day of last menses and the cycle length, which were both assessed before the GSST [20]. Only girls with a regular cycle between 21 and 35 days were included in the analyses concerning menstrual phase. Of the 295 girls who participated in the GSST and from whom blood was available, 234 (79%) reported to have a regular cycle. We defined the follicular phase as the period between the first day of the cycle and 14 days before the end of the cycle (n=125) and the luteal phase as the last 14 days of the cycle (n=109). We distinguished between non-habitual smokers and habitual smokers (≥ 1 cigarette/week). Smoking data was missing from 9 GSST participants. Physical development (PD) was assessed by self-report using the physical development scale [28]. Z-scores for PD were calculated for boys and girls separately (missing N=41). Socioeconomic status (SES) was calculated as the z-score of the mean of income, educational, and occupational level of both parents during the first assessment (missing N=13).

## Statistical analyses

Descriptives for age, PD, SES, ACTH IgG levels and antisocial behavior scores were computed, and independent t-tests were used to compare boys and girls, OC-users and non-OC-users. Spearman's Rho correlation coefficients were computed separately for boys and girls.

To study our main aim, the relationship between ACTH IgG and antisocial behavior, and between ACTH IgG and cortisol response, we used regression analyses. Linear regression analyses were conducted with ACTH IgG as independent and antisocial behavior as dependent variables, with sex, age and SES as covariates. We tested interaction of sex and ACTH IgG levels and stratified analyses if interaction terms were significant. The interaction between free and total ACTH IgG levels was also tested. Linear regression analyses were conducted with ACTH IgG as independent and cortisol measures (AUCincr or Recovery) as dependent variables, with sex, age, SES, PD smoking and OC use (in girls) as covariates. In additional analyses we used linear regression analysis to describe the associations between antisocial behavior and cortisol measures. Cortisol measures, ACTH IgG levels and antisocial behavior scores were transformed using natural logarithm before analyses. Missing values were excluded list-wise.

Within our sub-study on affinity of ACTH IgG, we conducted the following analyses: Independent sample t-tests were applied to assess differences between mean levels of total or ACTH-reactive IgG levels and affinity kinetics measures in adolescents with high or low total ACTH IgG levels for boys and girls separately.

Outliers in measures of affinity kinetics (Ka and Kd) were excluded before analyses (+/- 3x standard deviation, n=2).

## Results

### Descriptives

Girls had higher mean levels of free and total ACTH IgG than boys (table 1). ACTH IgG levels did not differ between GSST participants and non-participants, they also did not differ between OC-users and non-OC-users. The cortisol response to the GSST was blunted in OC-users, as shown earlier [20]. Free and total ACTH IgG levels correlated positively with each other (girls ρ=0.34; boys ρ=0.42) and with age (girls ρ=0.17 (total); boys ρ=0.09 (free)-0.22 (total)), with PD (ρ=0.08 (free)-0.12 (total)) in boys and with SES (ρ=-0.16 (total) and irregular menstruation (ρ=0.16, free) in girls (supplementary table 1).

**Table 1:** Descriptives

| | boys | | | girls | | |
|---|---|---|---|---|---|---|
| | **N** | **mean** | **SD** | **N** | **mean** | **SD** |
| Age (years) | 573 | 16.2 | 0.64 | 657 | 16.2 | 0.68 |
| Physical development | 554 | 0.01 | 0.97 | 635 | 0.00 | 0.99 |
| Socioeconomic status | 566 | 0.11 | 0.79 | 651 | 0.10 | 0.76 |
| Antisocial behavior (ASBQ) | 554 | 0.29** | 0.33 | 641 | 0.16 | 0.22 |
| Smoking (≥ 1 cigarette/week) | 158 (28.5%) | | | 204 (31.8%) | | |
| *ACTH immunoglobulins (OD)* | | | | | | |
| Free IgG | 573 | 0.59* | 0.44 | 657 | 0.67 [b] | 0.50 |
| Total IgG | 573 | 2.14** | 0.42 | 657 | 2.24 [b] | 0.39 |
| *HPA axis activity (nmol/l)* | | | | | | |
| AUCincr | 270 | 82.1* | 105.5 | 192 (no OC) | 70.4 [a] | 115.1 |
| | | | | 94 (OC) | 19.0 | 41.4 |
| Recovery | 277 | 1.8* | 2.1 | 294 | 1.2 [b] | 2.3 |

* p < 0.01, ** p < 0.001 difference between boys and girls

[a] p < 0.001 difference between girls taking OC and girls not taking OC

[b] no difference between girls taking OC and girls not taking OC

Abbreviations: ASBQ, Antisocial Behavior Questionnaire; ACTH, corticotropin; OD, optical density; HPA axis, hypothalamic-pituitary-adrenal axis; AUCincr, area under the curve during social stress test with respect to the starting value; OC, oral contraceptives ; SD, standard deviation

### ACTH IgG and antisocial behavior

In the model for antisocial behavior, the interaction between sex and free ACTH IgG was significant (β=0.17; p=0.02), therefore we stratified by gender. In boys, high

total ACTH IgG levels were associated with a higher score for antisocial behavior, and this association was stronger at higher levels of free ACTH IgG (table 2). The model improved significantly (-2 loglikelihood difference (LLH)= 5.1; 1 df; p=0.02) when the interaction between free and total ACTH IgG was included. In girls, high levels of free ACTH IgG were associated with a lower antisocial behavior score. The model did not improve after adding the interaction between free and total ACTH IgG (-2 LLH difference=0.3; 1 df; p=0.60). The level of total ACTH IgG was not related to antisocial behavior in girls. To explore relations with varying antisocial behaviors we carried out logistic regression analyses with groups of items of the ASBQ. The strongest relationships were with destruction of property, involvement in physical fights and theft (supplementary table 2).

**Table 2:** Associations between ACTH immunoglobulin (IgG) and antisocial behavior

| | Boys and Girls | | | Boys | | | Girls | | |
|---|---|---|---|---|---|---|---|---|---|
| | **β** | **95% CI** | **p** | **β** | **95% CI** | **p** | **β** | **95% CI** | **p** |
| Free ACTH IgG | -0.02 | -0.19; 0.09 | 0.50 | -0.64 | -1.36; 0.08 | 0.08 | -0.20 | -0.39; -0.01 | 0.04 |
| Total ACTH IgG | 0.04 | -0.18; 0.68 | 0.26 | 1.05 | 0.03; 2.07 | 0.04 | 0.33 | -0.29; 0.94 | 0.30 |
| Free x total ACTH IgG | | | | 1.03 | 0.13; 1.93 | 0.03 | | | |

Covariates in the model: age, socioeconomic status
Abbreviations: ACTH, corticotropin; CI, confidence interval

## ACTH IgG and stress-induced cortisol response

In the model for AUCincr, the interaction between sex and free ACTH IgG was significant (β=-0.94; p<0.01), and in the model for recovery, the interaction between sex and total ACTH IgG was significant (β=-1.62; p<0.01), hence we stratified by gender. Levels of free ACTH IgG were negatively associated with AUCincr in boys (β=-0.67; p<0.01, table 3). Total ACTH IgG level was associated with higher recovery of the cortisol response in girls (β=0.84; p=0.05, table 3). The associations between ACTH IgG and cortisol responses did not change after adding menstrual cycle stage as covariate. Unadjusted cortisol responses in boys and girls with low (first quartile) or high (third quartile) free and total ACTH IgG levels are depicted in figure

There was a trend towards a negative association between antisocial behavior and AUCincr (β=-0.13, 95% CI: -0.73; 0.01, p=0.08) in the total sample. The interaction between sex and antisocial behavior was not significant. The association with Recovery was not significant (β=-0.01, 95% CI: -0.09; 0.07, p=0.81).

**Table 3:** Associations between ACTH immunoglobulin (IgG) and HPA axis activity during social stress

| | AUCincr | | | Recovery | | |
|---|---|---|---|---|---|---|
| | β | 95% CI | p | β | 95% CI | p |
| Boys and girls | | | | | | |
| Total ACTH IgG | -0.09 | -1.12; 0.94 | 0.86 | -0.06 | -0.63; 0.51 | 0.84 |
| Free ACTH IgG | -0.04 | -0.45; 0.17 | 0.37 | 0.02 | -0.15; 0.18 | 0.86 |
| Boys | | | | | | |
| Total ACTH IgG | 0.07 | -1.27; 1.40 | 0.88 | -0.65 | -1.42; 0.13 | 0.10 |
| Free ACTH IgG | -0.67 | -1.10; -0.24 | <0.01 | -0.04 | -0.30; 0.21 | 0.78 |
| Girls | | | | | | |
| Total ACTH IgG | -0.38 | -1.01; 1.15 | 0.63 | 0.85 | -0.00; 1.70 | 0.05 |
| Free ACTH IgG | 0.31 | -0.11; 0.74 | 0.15 | 0.05 | -0.17; 0.28 | 0.63 |

Covariates in the model: age, socioeconomic status, physical development, smoking, OC use (girls)
Abbreviations: ACTH, corticotropin; AUCincr, area under the curve during social stress test with respect to the starting value; Recovery, recovery after social stress test; CI, confidence interval; OC, oral contraceptives

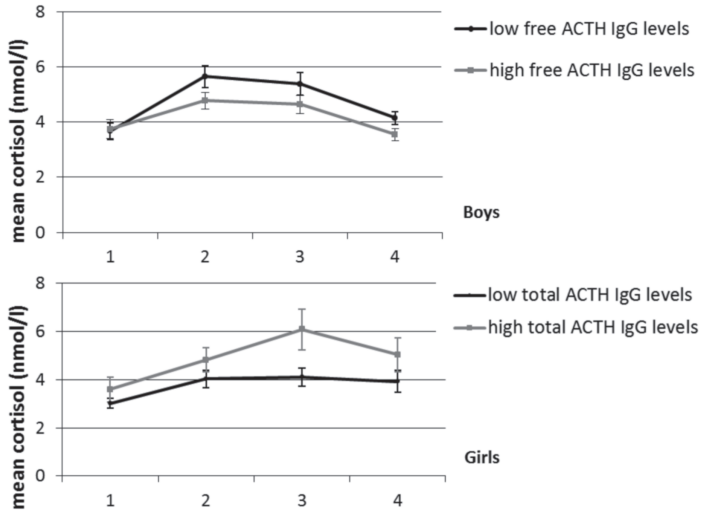


**Figure 1:** Cortisol levels during the social stress test in boys and girls with high and low free or total ACTH immunoglobulin (IgG) levels. Subjects with low ACTH IgG levels were defined as those in the first quartile, high ACTH IgG levels as those in the third quartile. Values are shown with standard errors.
Abbreviations: ACTH, corticotropin

## Affinity kinetics

There were no significant differences in age, PD and SES between boys or girls selected for high and low total ACTH IgG levels. ACTH-reactive IgM and IgA did not differ significantly between groups (figure 2b,c). Total IgG and IgM concentrations

were higher in girls with high ACTH IgG levels (figure 2d,e). Total IgA and ACTH peptide concentration did not differ between groups (figure 2f,h). As expected based on the positive association with total ACTH IgG in boys but not in girls, in the selected samples antisocial behavior scores were higher in boys with high total ACTH IgG levels (figure 2g). Free ACTH IgG levels were higher in girls with high total ACTH IgG levels (2i). The association rate constant (Ka) was lower in boys and girls with high compared to low total ACTH IgG (figure 2j). Dissociation rate constant (Kd) and affinity constant at equilibrium (KD) did not differ between groups (figure 2k, l). The model fit for affinity kinetics after applying the Langmuir (1:1) binding model had an average Chi² of 317 (SD=162).

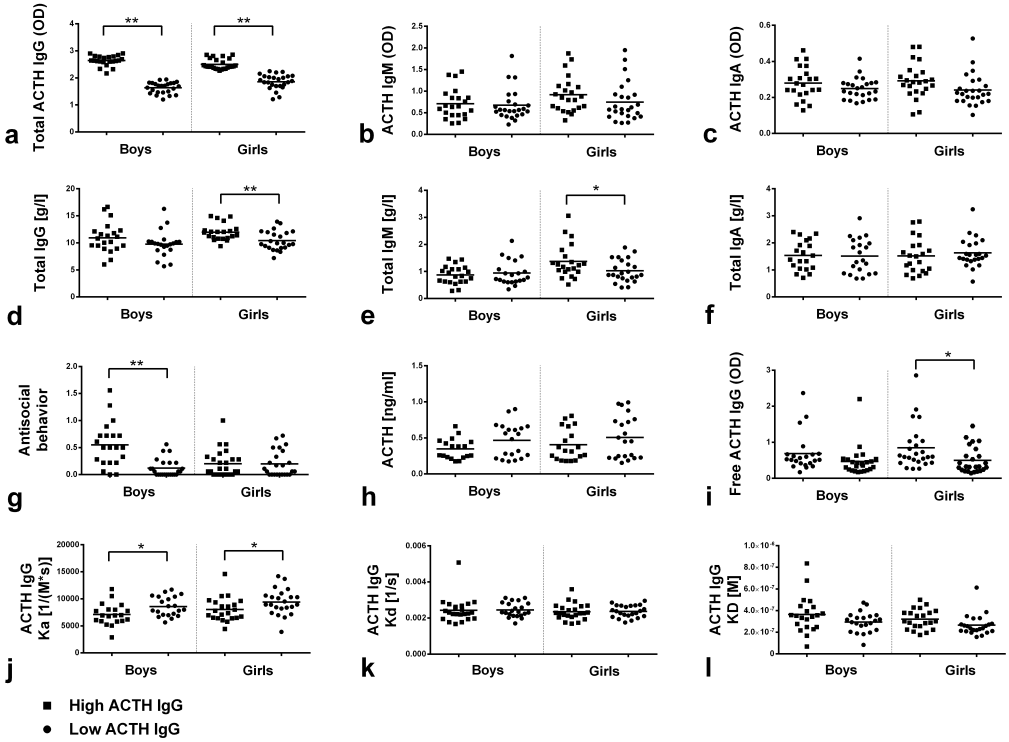


**Figure 2:** ACTH-reactive immunoglobulins (Ig), serum total Ig concentrations and affinity measures in selected samples with high or low total ACTH IgG. Comparison between boys and girls with high or low ACTH IgG levels: a) total ACTH IgG levels, these were used for selection; b) ACTH-reactive IgM levels; c) ACTH-reactive IgA levels; d) total IgG levels; e) total IgM levels; f) total IgA levels; g) antisocial behavior scores; h) ACTH peptide levels; i) free ACTH IgG levels; j) Ka of ACTH-reactive IgG; k) Kd of ACTH-reactive IgG; and l) KD of ACTH-reactive IgG. * $p < 0.05$, ** $p < 0.01$
Abbreviations: ACTH, corticotropin; OD, optical density; Ka, association rate constant; Kd, dissociation rate constant; KD, dissociation constant at equilibrium

## Discussion



Our study shows that serum levels of ACTH-reactive IgG are associated with antisocial behavior in both male and female adolescents from the general population, supporting the previous findings in adult imprisoned males. Furthermore, we found gender-specific associations between ACTH IgG levels and stress-induced cortisol responses.

Most studies on HPA axis activity and antisocial behavior support the hypoarousal theory, according to which antisocial behavior is related to lower cortisol levels in boys [4-7] and girls [17], as well as to lower cortisol responses to frustrating stressors [29,30] or a public speaking task [16]. In our study, boys with high antisocial behavior scores had higher total ACTH IgG levels. This relationship was stronger if they also had high levels of free ACTH IgG. Assuming a neutralizing role for ACTH immunoglobulins, this finding is in line with the hypoarousal theory. ACTH-reactive IgG could prevent ACTH from binding to melanocortin receptor 2, needed to induce cortisol release, resulting in low cortisol levels ('hypoarousal'). This sub-optimal physiological state has been linked to sensation-seeking behavior and higher levels of externalizing behavior in boys [4,5]. In support of a neutralizing function of ACTH IgG, in our study, high levels of free ACTH IgG were associated with a blunted cortisol response to stress in boys. The association between antisocial behavior and cortisol showed a similar pattern, there was a trend for a blunted cortisol response.

Two roles of ACTH IgG are plausible, transportation or neutralization. While our results so far suggest a neutralizing function, other studies have suggested a transport function for neuropeptide-reactive IgG [13]. Assuming that the main role for ACTH IgG would be transportation of ACTH, high levels of free ACTH IgG would be a sign for lower binding capacity of ACTH by ACTH IgG. The reason for such insufficient binding could be a low affinity of the antibody for ACTH. A result of this insufficient binding of ACTH by ACTH IgG would be impaired transportation, leading to a blunted cortisol response. For a better understanding of the functional mechanism of ACTH-reactive IgG, affinity measurements could be useful. Our affinity kinetics results showed no differences in affinity constants (KD) between subjects with high compared to low levels of ACTH IgG. But, we observed lower association rate constants (Ka) of ACTH IgG in adolescents with high ACTH IgG levels, suggesting an impaired ACTH binding in these subjects. Although we did not find differences in affinities, kinetic properties such as Ka may also affect biological processes. Further research is needed to understand the properties such as binding kinetics and the working mechanism of ACTH-reactive IgG.

It is unknown which factors influence ACTH IgG production and affinity properties. In animal models, levels of immunoglobulins against other neuropeptides increased after exposure to stress e.g. by food restriction [13,31]. Hence, a possible connection between high ACTH IgG levels, HPA axis dysregulation and antisocial behavior could be exposure to physical or psychosocial stress. Stressful life events can induce antisocial behavior, and vice versa [32]. This possible relation needs further testing.

Another possibility is the formation of ACTH IgG due to molecular mimicry. Sequence homology between ACTH and microbial proteins has been demonstrated [33].

Average levels of ACTH IgG were higher in girls than boys. This indicates that the mechanism of ACTH IgG synthesis is related to biological or environmental gender-specific factors. In accordance with this finding, higher mean concentrations of total IgG and IgM were reported in females compared to males, in both child and adult populations [34,35]. Regression analyses revealed different associations between ACTH IgG levels and antisocial behavior in girls versus boys. In girls, lower levels of free ACTH IgG were associated with higher antisocial behavior scores. This is contrary to the findings in boys. Furthermore, recovery during the GSST was positively associated with total ACTH IgG levels, while there was no relation between AUCincr and IgG levels in girls. Hence, not only the mechanism of formation, but also the role of ACTH IgG seems to be gender-specific. Presently, we cannot explain these discrepancies, but one possibility is that sex hormones are involved. Sex hormones have been implicated in immune system modulation, with estrogen stimulating humoral immunity [36]. They are also involved in HPA axis regulation [37], hence it is plausible that sex hormones modify ACTH IgG levels via both changes in the immune system and in the functioning of the HPA axis. One study found lower cortisol to DHEA ratios, higher free testosterone and lower SHBG levels in girls with aggressive conduct disorder compared to normal controls and girls with non-aggressive conduct disorder [38]. We found that girls with higher free ACTH IgG levels were more likely to have an irregular menstruation. Another possibility is, therefore, that not the sex hormones themselves influence levels of natural autoantibodies such as ACTH IgG, but that their regulation factors influence ACTH IgG production. The connection between stress, sex hormones and anti-neuropeptide immunoglobulin formation deserves further attention.

One limitation of this study was the cross-sectional design, which did not allow us to investigate changes of ACTH IgG levels over time. Therefore, we cannot establish causality. The associations found in our sample are weaker than those found in prisoners, probably due to less severe antisocial behavior and much lower levels of psychopathic traits in our general population sample. Some psychopathic traits are suggested to be specifically related to HPA axis reactivity, like callous unemotional traits [39,40]. We could not compare those specific traits in our study, but we found stronger relationships between ACTH IgG and severe items of the ASBQ (e.g. destruction of property and involvement in physical fights) than with less severe items (minor offenses). It is important to note that affinity kinetics were analyzed using the total serum IgG. Group differences in Ka were possibly influenced by ACTH IgG levels in the sera.

The strengths of our study are a large general population sample of both female and male adolescents, availability of detailed measures of the stress-induced cortisol response and potential confounders. Our large sample size allowed us to detect even weak associations between ACTH IgG levels and antisocial behavior and cortisol response, and to investigate gender-specific associations.

In conclusion, we showed that ACTH-reactive IgG levels are associated with both antisocial behavior and HPA axis reactivity in a large adolescent sample. The cortisol response is usually the only measure used to assess the relationship between HPA axis functioning and antisocial behavior. Yet, the cortisol response is influenced by numerous factors, such as sex, pubertal status, OC use and smoking. Even studies with adjustment for covariates show contrasting results. It is plausible that other biological factors fine-tune this association and that ACTH IgG may be one such factor modulating the link between antisocial behavior and cortisol levels.

Future research should focus on elucidating the working mechanism of ACTH IgG, for example by looking at antibody modulation of melanocortin receptor binding, and by repeated measurements of cortisol, ACTH peptide and ACTH IgG, to study concurrent dynamics of these components of the HPA axis. Longitudinal studies are needed to assess causality of the association between ACTH IgG and antisocial behavior. Knowing more about the working mechanism and function of ACTH IgG may help to better understand the complex biological pathways underlying HPA axis (dys)regulation and behavioural problems.

## References


1. van Goozen SH, Fairchild G, Snoek H, Harold GT (2007) The evidence for a neurobiological model of childhood antisocial behavior. Psychol Bull 133: 149-182.
2. Alink LR, van Ijzendoorn MH, Bakermans-Kranenburg MJ, Mesman J, Juffer F, et al. (2008) Cortisol and externalizing behavior in children and adolescents: mixed meta-analytic evidence for the inverse relation of basal cortisol and cortisol reactivity with externalizing behavior. Dev Psychobiol 50: 427-450.
3. Ruttle PL, Shirtcliff EA, Serbin LA, Fisher DB, Stack DM, et al. (2011) Disentangling psychobiological mechanisms underlying internalizing and externalizing behaviors in youth: longitudinal and concurrent associations with cortisol. Horm Behav 59: 123-132.
4. Shoal GD, Giancola PR, Kirillova GP (2003) Salivary cortisol, personality, and aggressive behavior in adolescent boys: a 5-year longitudinal study. J Am Acad Child Adolesc Psychiatry 42: 1101-1107.
5. McBurnett K, Lahey BB, Rathouz PJ, Loeber R (2000) Low salivary cortisol and persistent aggression in boys referred for disruptive behavior. Arch Gen Psychiatry 57: 38-43.
6. Poustka L, Maras A, Hohm E, Fellinger J, Holtmann M, et al. (2010) Negative association between plasma cortisol levels and aggression in a high-risk community sample of adolescents. J Neural Transm 117: 621-627.
7. Haltigan JD, Roisman GI, Susman EJ, Barnett-Walker K, Monahan KC (2011) Elevated trajectories of externalizing problems are associated with lower awakening cortisol levels in midadolescence. Dev Psychol 47: 472-478.
8. Klimes-Dougan B, Hastings PD, Granger DA, Usher BA, Zahn-Waxler C (2001) Adrenocortical activity in at-risk and normally developing adolescents: individual differences in salivary cortisol basal levels, diurnal variation, and responses to social challenges. Dev Psychopathol 13: 695-719.
9. van Bokhoven I, Van Goozen SH, van Engeland H, Schaal B, Arseneault L, et al. (2005) Salivary cortisol and aggression in a population-based longitudinal study of adolescent males. J Neural Transm 112: 1083-1096.
10. McBurnett K, Raine A, Stouthamer-Loeber M, Loeber R, Kumar AM, et al. (2005) Mood and hormone responses to psychological challenge in adolescent males with conduct problems. Biol Psychiatry 57: 1109-1116.
11. Fetissov SO, Hallman J, Nilsson I, Lefvert AK, Oreland L, et al. (2006) Aggressive behavior linked to corticotropin-reactive autoantibodies. Biol Psychiatry 60: 799-802.
12. Haller J, Kruk MR (2006) Normal and abnormal aggression: human disorders and novel laboratory models. Neurosci Biobehav Rev 30: 292-303.
13. Sinno MH, Do Rego JC, Coeffier M, Bole-Feysot C, Ducrotte P, et al. (2009) Regulation of feeding and anxiety by alpha-MSH reactive autoantibodies. Psychoneuroendocrinology 34: 140-149.
14. Deloumeau A, Bayard S, Coquerel Q, Dechelotte P, Bole-Feysot C, et al. (2010) Increased immune complexes of hypocretin autoantibodies in narcolepsy. PLoS zOne 5: e13320.
15. Gustaw KA, Garrett MR, Lee HG, Castellani RJ, Zagorski MG, et al. (2008) Antigen-antibody dissociation in Alzheimer disease: a novel approach to diagnosis. J Neurochem 106: 1350-1356.
16. Popma A, Jansen LM, Vermeiren R, Steiner H, Raine A, et al. (2006) Hypothalamus pituitary adrenal axis and autonomic activity during stress in delinquent male adolescents and controls. Psychoneuroendocrinology 31: 948-957.
17. Pajer K, Gardner W, Rubin RT, Perel J, Neal S (2001) Decreased cortisol levels in adolescent girls with conduct disorder. Arch Gen Psychiatry 58: 297-302.

18. Gaillard RC, Spinedi E (1998) Sex- and stress-steroids interactions and the immune system: evidence for a neuroendocrine-immunological sexual dimorphism. Domest Anim Endocrinol 15: 345-352.

19. Huisman M, Oldehinkel AJ, de Winter A, Minderaa RB, de Bildt A, et al. (2008) Cohort profile: the Dutch 'TRacking Adolescents' Individual Lives' Survey'; TRAILS. Int J Epidemiol 37: 1227-1235.

20. Bouma EM, Riese H, Ormel J, Verhulst FC, Oldehinkel AJ (2009) Adolescents' cortisol responses to awakening and social stress; effects of gender, menstrual phase and oral contraceptives. The TRAILS study. Psychoneuroendocrinology 34: 884-893.

21. Kudielka BM, Schommer NC, Hellhammer DH, Kirschbaum C (2004) Acute HPA axis responses, heart rate, and mood changes to psychosocial stress (TSST) in humans at different times of day. Psychoneuroendocrinology 29: 983-992.

22. Kirschbaum C, Pirke KM, Hellhammer DH (1993) The 'Trier Social Stress Test'-a tool for investigating psychobiological stress responses in a laboratory setting. Neuropsychobiology 28: 76-81.

23. Sondeijker FE, Ferdinand RF, Oldehinkel AJ, Veenstra R, Tiemeier H, et al. (2007) Disruptive behaviors and HPA-axis activity in young adolescent boys and girls from the general population. J Psychiatr Res 41: 570-578.

24. Moffitt TE, Silva PA (1988) Neuropsychological deficit and self-reported delinquency in an unselected birth cohort. J Am Acad Child Adolesc Psychiatry 27: 233-240.

25. Kirschbaum C, Read GF, Hellhammer D (1992) Assessment of hormones and drugs in saliva in biobehavioral research. Seattle: Hogrefe & Huber. 356 p. p.

26. Janssens KA, Oldehinkel AJ, Verhulst FC, Hunfeld JA, Ormel J, et al. (2012) Symptom-specific associations between low cortisol responses and functional somatic symptoms: the TRAILS study. Psychoneuroendocrinology 37: 332-340.

27. Fetissov SO (2011) Neuropeptide autoantibodies assay. Methods Mol Biol 789: 295-302.

28. Williams JM, Dunlop LC (1999) Pubertal timing and self-reported delinquency among male adolescents. J Adolesc 22: 157-171.

29. Snoek H, Van Goozen SH, Matthys W, Buitelaar JK, van Engeland H (2004) Stress responsivity in children with externalizing behavior disorders. Dev Psychopathol 16: 389-406.

30. van Goozen SH, Matthys W, Cohen-Kettenis PT, Buitelaar JK, van Engeland H (2000) Hypothalamic-pituitary-adrenal axis and autonomic nervous system activity in disruptive children and matched controls. J Am Acad Child Adolesc Psychiatry 39: 1438-1445.

31. Coquerel Q, Sinno MH, Boukhettala N, Coeffier M, Terashi M, et al. (2012) Intestinal inflammation influences alpha-MSH reactive autoantibodies: relevance to food intake and body weight. Psychoneuroendocrinology 37: 94-106.

32. Kim KJ, Conger RD, Elder GH, Jr., Lorenz FO (2003) Reciprocal influences between stressful life events and adolescent internalizing and externalizing problems. Child Dev 74: 127-143.

33. Fetissov SO, Hamze Sinno M, Coeffier M, Bole-Feysot C, Ducrotte P, et al. (2008) Autoantibodies against appetite-regulating peptide hormones and neuropeptides: putative modulation by gut microflora. Nutrition 24: 348-359.

34. Gonzalez-Quintela A, Alende R, Gude F, Campos J, Rey J, et al. (2008) Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. Clin Exp Immunol 151: 42-50.

35. Stoop JW, Zegers BJ, Sander PC, Ballieux RE (1969) Serum immunoglobulin levels in healthy children and adults. Clin Exp Immunol 4: 101-112.

36. Magiakou MA, Mastorakos G, Webster E, Chrousos GP (1997) The hypothalamic-pituitary-adrenal axis and the female reproductive system. Ann N Y Acad Sci 816: 42-56.

37. Chrousos GP, Torpy DJ, Gold PW (1998) Interactions between the hypothalamic-pituitary-adrenal axis and the female reproductive system: clinical implications. Ann Intern Med 129: 229-240.

38. Pajer K, Tabbah R, Gardner W, Rubin RT, Czambel RK, et al. (2006) Adrenal androgen and gonadal hormone levels in adolescent girls with conduct disorder. Psychoneuroendocrinology 31: 1245-1256.

39. Loney BR, Butler MA, Lima EN, Counts CA, Eckel LA (2006) The relation between salivary cortisol, callous-unemotional traits, and conduct problems in an adolescent non-referred sample. J Child Psychol Psychiatry 47: 30-36.

40. O'Leary MM, Loney BR, Eckel LA (2007) Gender differences in the association between psychopathic personality traits and cortisol response to induced stress. Psychoneuroendocrinology 32: 183-191.

## Supplementary information: Results

**Supplementary table 1** Correlations between ACTH immunoglobulins (Ig), cortisol measures, antisocial behavior and covariates in boys and girls

| | ASB | Free ACTH IgG | Total ACTH IgG | AUCincr | Recovery | Age | SES | PD | Irreg. Mens. |
|---|---|---|---|---|---|---|---|---|---|
| ASB | 1 | -0.07 | 0.03 | -0.20** | -0.07 | -0.03 | -0.13** | -0.06 | 0.00 |
| Free ACTH IgG | 0.03 | 1 | 0.34** | 0.10 | 0.07 | -0.01 | -0.06 | -0.05 | 0.16** |
| Total ACTH IgG | 0.05 | 0.42** | 1 | 0.02 | 0.07 | 0.17** | -0.16** | -0.06 | 0.07 |
| AUCincr | -0.07 | -0.15* | -0.07 | 1 | 0.39** | -0.04 | 0.15* | -0.04 | 0.17** |
| Recovery | -0.06 | -0.05 | -0.12 | 0.65** | 1 | -0.01 | -0.07 | 0.05 | 0.01 |
| Age | -0.11** | 0.09* | 0.22** | 0.03 | -0.01 | 1 | -0.03 | 0.33** | -0.01 |
| SES | -0.12** | 0.01 | -0.02 | 0.10 | 0.10 | 0.10* | 1 | 0.10** | 0.06 |
| PD | -0.04 | 0.08* | 0.12** | 0.14* | 0.04 | 0.28** | 0.02 | 1 | -0.05 |

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

Spearman's Rank correlation was used; above diagonal: girls; below diagonal: boys

Abbreviations: ASB, antisocial behavior; ACTH, corticotropin; AUCincr, area under the curve during social stress test with respect to the starting value; Recovery, recovery after social stress test; SES, socioeconomic status; PD, physical development

**Supplementary table 2** ASBQ groups of items and associations with ACTH IgG

| | Boys and Girls | | | Boys | | | Girls | | |
|---|---|---|---|---|---|---|---|---|---|
| | N (%) | OR | 95% CI | N (%) | OR | 95% CI | N (%) | OR | 95% CI |
| Destruction of property (at home/ school/ in public, vandalism e.g. graffiti) | | | | | | | | | |
| Free ACTH IgG | 381 (32%) | **0.29** | **0.12; 0.71** | 244 (44.2%) | **0.3** | **0.09; 0.98** | 137 (21.5%) | **0.22** | **0.05; 0.97** |
| Total ACTH IgG | 381 (32%) | **4.48** | **1.31; 15.34** | 244 (44.2%) | **5.54** | **1.08; 28.50** | 137 (21.5%) | 3.72 | 0.53; 26.06 |
| Free x total ACTH IgG | 381 (32%) | **4.42** | **1.48; 13.20** | 244 (44.2%) | **4.88** | **1.13; 21.18** | 137 (21.5%) | 4.92 | 0.88; 27.38 |
| Involved in physical fights (at school/ home/ on street) | | | | | | | | | |
| Free ACTH IgG | 439 (37%) | 0.64 | 0.27; 1.52 | 248 (45.3%) | 0.34 | 0.10; 1.12 | 191 (29.8%) | 1.3 | 0.35; 4.88 |
| Total ACTH IgG | 439 (37%) | 2.45 | 0.77; 7.73 | 248 (45.3%) | **5.41** | **1.03; 28.54** | 191 (29.8%) | 1.19 | 0.23; 6.10 |
| Free x total ACTH IgG | 439 (37%) | 2.04 | 0.71; 5.85 | 248 (45.3%) | **5.31** | **1.18; 23.94** | 191 (29.8%) | 0.78 | 0.16; 3.80 |
| Major offenses (sold/received stolen goods, threatened someone with violence, member of a gang, set fire, carried a weapon or used it in a fight) | | | | | | | | | |
| Free ACTH IgG | 227 (19%) | 0.58 | 0.20; 1.65 | 92 (16.7%) | 0.9 | 0.18; 4.62 | 135 (20.5%) | 0.35 | 0.08; 1.44 |
| Total ACTH IgG | 227 (19%) | 3.11 | 0.77; 12.56 | 92 (16.7%) | 3.41 | 0.39; 29.84 | 135 (20.5%) | 3.13 | 0.48; 20.32 |
| Free x total ACTH IgG | 227 (19%) | 2.15 | 0.61; 7.58 | 92 (16.7%) | 1.21 | 0.16; 8.94 | 135 (20.5%) | 3.97 | 0.74; 21.35 |
| Minor offenses (deflated tires, made phone calls, dodged fares on public transport, suspended from class) | | | | | | | | | |
| Free ACTH IgG | 908 (76.2%) | 1.06 | 0.41; 2.76 | 452 (82%) | 1.19 | 0.22; 6.63 | 456 (71.3%) | 1.08 | 0.31; 3.74 |
| Total ACTH IgG | 908 (76.2%) | 0.98 | 0.27; 3.49 | 452 (82%) | 0.67 | 0.06; 7.37 | 456 (71.3%) | 1.26 | 0.26; 6.09 |
| Free x total ACTH IgG | 908 (76.2%) | 0.9 | 0.28; 2.92 | 452 (82%) | 1.81 | 0.21; 15.53 | 456 (71.3%) | 0.59 | 0.13; 2.64 |

**Supplementary table 2** (continued)

| | Boys and Girls | | | Boys | | | Girls | | |
|---|---|---|---|---|---|---|---|---|---|
| | N (%) | OR | 95% CI | N (%) | OR | 95% CI | N (%) | OR | 95% CI |
| Theft (stealing / shoplifting) | | | | | | | | | |
| Free ACTH IgG | 234 (19.6%) | 0.82 | 0.29; 2.32 | 165 (29.9%) | 0.33 | 0.09; 1.19 | 69 (10.8%) | 3.18 | 0.55; 18.38 |
| Total ACTH IgG | 234 (19.6%) | 1.15 | 0.28; 4.65 | 165 (29.9%) | **7.29** | **1.19; 44.77** | 69 (10.8%) | **0.06** | **0.006; 0.53** |
| Free x total ACTH IgG | 234 (19.6%) | 3.7 | 0.40; 5.14 | 165 (29.9%) | 4.83 | 0.97; 23.93 | 69 (10.8%) | 0.24 | 0.025; 2.32 |

Covariates: sex (full sample), age, socioeconomic status, physical development

In bold: p<0.0N represents the number of adolescents that said yes to one or more of the items in the group, % represents valid percent (excluding missing values (N missing (boys and girls)=37-42 depending on group)

Abbreviations: ASBQ, antisocial behavior questionnaire; OR, odds ratio; CI, confidence interval; ACTH, corticotropin; IgG, immunoglobulin G

# chapter 3

## Are immunoglobulins against melanocortin peptides associated with internalizing problems in adolescents? The TRAILS study.

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## Abstract

Internalizing problems such as anxiety and depression are common in adolescence, and are related to a dysregulation of biological stress responses. Immunoglobulins (Ig) reactive with the melanocortin peptides alpha-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) are new promising factors that could further our understanding of biological mechanisms underlying internalizing problems. We investigated in a large sample of adolescents from the general population whether α-MSH- and ACTH-reactive Ig levels were associated with internalizing problems.

ELISA was used to measure α-MSH IgG, α-MSH IgM and free and total ACTH IgG levels in 1230 Dutch adolescents (15-18 years). Internalizing problems were defined as the mean of the 'Withdrawn/depressed' and 'Anxious/depressed' scales of the Youth Self-Report. We adjusted for the influence of potential covariates – age, sex, socioeconomic status, body mass index and infections – on the association between Ig levels and internalizing problems.

In girls but not in boys, ACTH IgG levels were associated with internalizing problems ($\beta_{free}$= -0.51, $p<0.01$; $\beta_{total}$=-0.16, $p<0.01$). These associations were specific for the 'Anxious/depressed' symptom scale. α-MSH IgG and IgM levels were not associated with internalizing problems.

ACTH-reactive IgG levels are associated with internalizing problems in girls, suggesting their involvement in the pathophysiology of anxiety and depression. Their mechanism of action requires further study.

## Introduction



Internalizing problems, i.e., symptoms of anxiety and depression, are common in adolescence, and associated with adverse long-term effects such as later psychopathology [1,2], alcohol abuse [3], educational underachievement [4] and hypertension [5]. To improve diagnoses and treatments, there is a need to identify biological factors related to the onset, development and mechanism of internalizing problems.

The melanocortin peptides adrenocorticotropic hormone (ACTH) and α-melanocyte stimulating hormone (α-MSH) are important regulators of physiological functions, including emotion and behavior [6-9]. Studies in rats have shown that the activation of the melanocortin receptor 4 (MC4R) by α-MSH elicits anxiety-like behavior and anhedonia after exposure to stress [7-9]. Stress-induced activation of the melanocortin system is accompanied by activation of the hypothalamic-pituitary-adrenal (HPA) axis via ACTH [10-12]. The HPA axis is an important regulator of the stress response, and dysregulation of the HPA axis has been observed in patients with internalizing problems such as major depression and panic disorder [13,14]. In adolescents, depression has been linked to both *hyper*cortisolism and *hypo*cortisolism [15,16], depending on the chronicity of depressive symptoms [16]. However, the biological mechanisms underlying the dysregulation of the HPA axis in individuals with emotional problems are complex and not yet fully understood. New promising factors that could further this understanding are Immunoglobulins (Ig) that bind melanocortin peptides.

Melanocortin- reactive Ig circulate naturally in the blood as free molecules or bound to their respective antigens (ACTH or α-MSH). Studies in both rats and humans have suggested that ACTH-reactive and α-MSH-reactive Ig play a role in regulating emotion and behavior [17-20]. So far, studies have focused on specific patient groups and animal models, used small sample sizes and did not adjust for any potential covariates. Hence, it is not known whether melanocortin-reactive Ig are involved in regulating emotion and behavior in the general population. Our previous finding that ACTH IgG levels were associated with both antisocial behavior and HPA axis reactivity in adolescents [21] suggests that ACTH IgG may play a role in HPA axis regulation or dysregulation and may thereby be related to anxiety and depression.

The aim of this study was to examine the relationship between ACTH and α-MSH-reactive Ig levels and internalizing problems in a large sample of adolescents from the general population. We studied this association cross-sectionally, expecting that higher Ig levels would be related to more internalizing problems, as shown previously in animals and adults [18-20]. We further explored whether this relationship was stronger with symptoms of anxious or withdrawn behavior, and whether it was different in boys and girls.

## Methods

### Participants

The TRacking Adolescents' Individual Lives Survey (TRAILS) is a cohort study in the Netherlands that follows young adolescents into adulthood [22]. Measurements consist of validated questionnaires, interviews and biological measures. The study was approved by the National Dutch Medical Ethics Committee. The first assessment wave (T1) ran from March 2001 to July 200

During T1, 2230 children were enrolled (response rate 76.0%, 10-12 years, 50.8% girls) [23], 1816 of whom participated in T3 (response 81.4%, 15-18 years, 52.3% girls, September 2005 to December 2007). Blood serum for Ig analyses was available from 1230 participants (68% of the total sample at T3, 15-18 years, 53.4% girls), and was taken at T

The study participants who agreed to give blood and therefore were included in our study did not differ from those who did not give blood with respect to internalizing problems, physical development or body mass index (BMI) at T

Participants who gave blood had a higher socioeconomic status (M=0.10, SE=0.02) than those who did not (M=-0.24, SE=0.03), $p<0.01$, they were slightly younger (M=16.2, SE=0.02) than the others (M=16.5, SE=0.03), $p<0.01$, and more girls than boys agreed to give blood. All study participants provided written consent.

### Internalizing problems

To assess internalizing problems, we used the Youth Self-Report (YSR)[24]. The items of the YSR are rated on a three-point scale (0= not true, 1= somewhat or sometimes true, 2= very true or often true) and refer to the past six months. As a measure for 'Internalizing Problems' (21 items, Chronbach's $\alpha$ =0.88), we combined the items of the two scales 'Anxious/depressed' (13 items, Chronbach's $\alpha$ =0.84; e.g., 'I cry a lot', 'I am afraid of going to school', 'I am nervous or tense', 'I worry a lot') and 'Withdrawn/depressed' (8 items, Chronbach's $\alpha$ =0.74, e.g., 'There is very little that I enjoy', 'I would rather be alone than with others', 'I am unhappy, sad or depressed'). We did not include the scale 'Somatic problems', as somatic problems may be an underlying reason for elevated Ig levels and thereby could interfere with our analyses. Data on internalizing problems was missing for 33 participants.

### Immunoglobulin measurements

We chose to measure 'free' (unbound) and 'total' ACTH IgG levels. This method provides a rough estimate of relative affinity of ACTH IgG, with free ACTH IgG having a lower affinity than bound ACTH IgG, present in the total IgG fraction [25]. We measured both free IgG and free IgM against $\alpha$-MSH, as previous studies have shown that these were both related to behavioural problems in patients with eating disorders [18].

Ig against ACTH and α-MSH were measured in serum using an enzyme-linked immunosorbent assay (ELISA) [25]. Briefly, 96-well plates (Nunc Immunoplate, Rochester, NY) were coated with 2mg/ml ACTH or α-MSH (Bachem AG, Bubendorf, Switzerland) in 0.5M $Na_2CO_3$, 0.5M $NaHCO_3$, pH 9.6, and incubated for 72 hours at 4°C. Plates were washed using phosphate-buffered saline (PBS, 0.05% Tween20, pH 7.4). Serum was diluted 1:100 in either physiological buffer (PBS, 0.02% sodium azide, pH 7.4) to measure free ACTH IgG, and free α-MSH IgG and IgM, or in 3M NaCl, 1.5M glycine (pH 8.9), creating a dissociative condition to measure total ACTH IgG levels. After incubation at 4°C overnight, plates were washed and incubated for 3h with alkaline phosphatase-conjugated secondary antibody (anti-human IgG or IgM; Jackson ImmunoResearch, West Grove, PA). The reaction was developed for 40min with 150µl p-Nitrophenyl phosphate substrate solution (Sigma, St Louis, MO) at 1mg/ml in 0.1M Tris-HCl, 0.1M NaCl, 5mM $MgCl_2$-$6H_2O$, pH 9.

100µl NaOH (2M) was added to stop the reaction and optical density (OD) was measured at 405nm in an ELISA plate reader. We subtracted the values for blank samples, i.e., containing only the buffer and no serum, from serum measurements. The intra-assay coefficient of variation for IgG and IgM measurements was on average 4.8%, the inter-assay coefficient 6.5%.

## Covariates

We considered the following variables as covariates: socioeconomic status, general inflammation marker high-sensitivity C-reactive protein (hs-CRP), season of venipuncture, antibodies against infectious agents, physical development, and BMI. Socioeconomic status was calculated as the Z-score of the mean of income, educational, and occupational levels of both parents at T1 [26] (missing n=13). Hs-CRP serum concentrations were measured by nephelometry using a Dade Behring BN2 Nephelometer [27] (missing n=5). The measurement of concentrations of antibodies against infectious agents: Influenza A and B, human herpesvirus 6 (HHV-6), herpes simplex virus 1 and 2 (HSV-1 and HSV-2), Epstein-Barr Virus (EBV) and *Toxoplasma gondii*, has been described previously [28] (missing n=12). Physical development was assessed by self-report using the physical development scale [29]. Z-scores for physical development were calculated for boys and girls separately (missing n=41). BMI was calculated as weight in kg divided by height in $m^2$ (missing n=29).

## Statistical analyses

To address our main aim, we used linear regression models with Ig levels as independent variables and internalizing problems as dependent variable. We tested the influence of potential covariates and included them in the model if they changed the regression coefficients of the independent variables. Missing values were excluded list-wise. Based on previous sex-specific findings regarding ACTH IgG and cortisol responses [21], we stratified analyses for sex. We tested the interaction term of free and total ACTH IgG, as the free ACTH IgG are a fraction of the total

ACTH IgG, and the interplay of low and high affinity ACTH IgG could be important for their functioning [25]. To analyze whether associations were specific for either 'Anxious/depressed' or 'Withdrawn/depressed' symptoms, we performed additional linear regression analyses with the YSR scale 'Anxious/depressed', correcting for 'Withdrawn/depressed' and vice versa. We corrected our analyses for multiple testing using the Bonferroni method.

## Results

### Internalizing Problems and Ig levels

Girls had higher mean free and total ACTH IgG and free α-MSH IgG and IgM levels than boys. Girls also reported more internalizing problems than boys (Table 1).

**Table 1:** Descriptives

| | boys | | | girls | | |
|---|---|---|---|---|---|---|
| | **N** | **mean** | **SE** | **N** | **mean** | **SE** |
| Age (years) | 573 | 16.2 | 0.03 | 657 | 16.2 | 0.03 |
| Socioeconomic status (Z-score) | 566 | 0.11 | 0.03 | 651 | 0.10 | 0.03 |
| Physical development (Z-score) | 554 | 0.01 | 0.04 | 635 | 0.00 | 0.04 |
| Body mass index (kg/m$^2$) | 562 | 20.9* | 0.13 | 639 | 21.7 | 0.12 |
| Immunoglobulin levels (OD) | | | | | | |
| Free ACTH IgG | 573 | 0.59* | 0.02 | 657 | 0.67 | 0.02 |
| Total ACTH IgG | 573 | 2.14* | 0.02 | 657 | 2.24 | 0.02 |
| α-MSH IgG | 573 | 0.64* | 0.02 | 657 | 0.72 | 0.02 |
| α-MSH IgM | 573 | 0.48* | 0.02 | 657 | 0.62 | 0.02 |
| Internalizing problems (YSR) | 566 | 0.26* | 0.01 | 647 | 0.40 | 0.01 |
| Anxious/ depressed (YSR) | 556 | 0.19* | 0.01 | 641 | 0.39 | 0.01 |
| Withdrawn/ depressed (YSR) | 556 | 0.32* | 0.01 | 641 | 0.42 | 0.01 |

* p-value < 0.001 (significant after Bonferroni correction for 11 tests), comparison between boys and girls
Abbreviations: ACTH, adrenocorticotropic hormone; α-MSH, alpha-melanocyte stimulating hormone; SE, standard error; OD, optical density; YSR, Youth Self-Report

Free and total ACTH IgG levels and their interaction term were significantly associated with internalizing problems (Table 2). Stratification by sex revealed that this relationship was significant only in girls. The main effects and the significant interaction between free and total ACTH IgG combined indicate that total ACTH IgG levels are positively associated with internalizing problems, but only in the presence of high levels of free ACTH IgG (Figure 1A). The association is negative when levels of free ACTH IgG are low (Figure 1B). These associations remained significant after correction for multiple testing (correction for nine tests, resulting in a new p-value of 0.006). ACTH IgG levels were not significantly associated with internalizing problems in boys (Table 2).

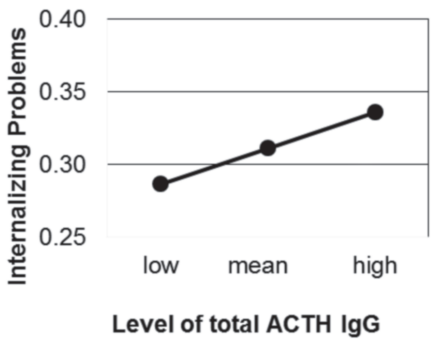


Figure 1A

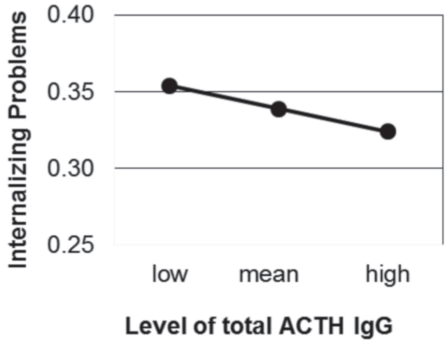


Figure 1B

**Figure 1:** Associations between total ACTH IgG levels and internalizing problems in girls at high levels of free ACTH IgG (Figure 1A) and at low levels of free ACTH IgG (Figure 1B).
Adjusted for age and socioeconomic status.
Low = mean-1SD, High = mean+1SD (for total ACTH IgG or free ACTH IgG)

**Table 2A:** Associations between ACTH-reactive IgG levels and internalizing problems

| | Internalizing problems | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total | | | Boys | | | Girls | | |
| | β | 95% CI | p | β | 95% CI | p | β | 95% CI | p |
| Free ACTH IgG | -0.30 | -0.52; -0.09 | 0.01 | -0.05 | -0.34; 0.24 | 0.75 | **-0.51** | **-0.83; -0.19** | **0.002** |
| Total ACTH IgG | -0.08 | -0.14; -0.01 | 0.02 | 0.01 | -0.07; 0.09 | 0.84 | **-0.16** | **-0.25; -0.06** | **0.002** |
| Free x total ACTH IgG | 0.12 | 0.03; 0.21 | 0.01 | 0.01 | -0.11; 0.13 | 0.85 | **0.21** | **0.08; 0.34** | **0.002** |

Adjusted for: sex (total sample), age and socioeconomic status; Statistically significant (p<0.006) associations are displayed in bold.

**Table 2B:** Associations between α-MSH-reactive IgG and IgM levels and internalizing problems

| | Internalizing problems | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total | | | Boys | | | Girls | | |
| | β | 95% CI | p | β | 95% CI | p | β | 95% CI | p |
| α-MSH IgG | 0.003 | -0.02; 0.03 | 0.85 | 0.003 | -0.03; 0.04 | 0.88 | 0.003 | -0.04; 0.04 | 0.87 |
| α-MSH IgM | 0.001 | -0.03; 0.04 | 0.94 | -0.007 | -0.05; 0.04 | 0.75 | 0.006 | -0.04; 0.06 | 0.81 |

Adjusted for: sex (total sample), age and socioeconomic status.
Abbreviations: ACTH, adrenocorticotropic hormone; α-MSH, alpha-melanocyte stimulating hormone; Ig, immunoglobulin; CI, confidence interval

The association between ACTH IgG and anxious/depressed symptoms in girls remained after adjusting for withdrawn/depressed symptoms, although it was not significant after correction for multiple testing (correction for 12 tests, resulting in a new p-value of 0.004). Withdrawn/depressed symptoms were not associated with ACTH IgG after adjustment for anxious/depressed symptoms (Table 3).

α-MSH IgG and IgM levels were not associated with internalizing problems (Table 2). This did not change after adjustment for any of the potential covariates.

**Table 3:** ACTH-reactive Ig levels in relation to the scales 'Anxious/depressed' and 'Withdrawn/depressed'

| | Boys | | | Girls | | |
|---|---|---|---|---|---|---|
| | **β** | **95% CI** | **p** | **β** | **95% CI** | **p** |
| Anxious/ depressed[a] | | | | | | |
| Free ACTH IgG | 0.10 | -0.12; 0.31 | 0.38 | -0.28 | -0.53; -0.03 | 0.03 |
| Total ACTH IgG | 0.05 | -0.01; 0.11 | 0.10 | -0.09 | -0.16; -0.01 | 0.02 |
| Free x total ACTH IgG | -0.04 | -0.13; 0.05 | 0.35 | 0.12 | 0.02; 0.22 | 0.02 |
| Withdrawn/ depressed[b] | | | | | | |
| Free ACTH IgG | -0.16 | -0.46; 0.13 | 0.28 | -0.03 | -0.28; 0.23 | 0.85 |
| Total ACTH IgG | -0.05 | -0.14; 0.03 | 0.18 | -0.01 | -0.08; 0.07 | 0.88 |
| Free x total ACTH IgG | 0.07 | -0.06; 0.19 | 0.31 | 0.00 | -0.10; 0.11 | 0.95 |

Abbreviations: ACTH, adrenocorticotropic hormone; Ig, immunoglobulin; CI, confidence interval
Adjusted for: age and socioeconomic status, [a] adjusted for 'Withdrawn/depressed',
[b] adjusted for 'Anxious/depressed'.

## Covariates

Correlations of potential covariates with melanocortin peptide-reactive Ig were weak and few remained significant after correction for 64 tests ($p<0.0008$, Table 4). Total ACTH IgG levels correlated with age, socioeconomic status, BMI, and physical development, though correlations with BMI and physical development were not statistically significant after Bonferroni correction. Season of venipuncture was related to Ig levels, e.g. total ACTH IgG were higher in autumn (M=2.23, SE=0.02) than in winter (M=2.14, SE=0.02), F=3.737, p=0.0

But adding BMI, physical development, or season of venipuncture to the model did not change the regression coefficients of the association between ACTH IgG levels and internalizing problems. Similarly, while melanocortin peptide-reactive Ig were correlated with IgG against pathogens, especially HSV-2 and influenza A, the inclusion of any pathogen-specific IgG in the regression models did not change the association between melanocortin Ig levels and internalizing problems. Because hs-CRP concentration did not correlate with melanocortin peptide Ig levels, it was not added to the regression models. Our final models included sex, age and socioeconomic status as covariates.

**Table 4:** Correlations between ACTH and α-MSH Ig levels and covariates

| | Free ACTH IgG | Total ACTH IgG | α-MSH IgM | α-MSH IgG |
|---|---|---|---|---|
| Free ACTH IgG | | | | |
| Total ACTH IgG | .38* | | | |
| α-MSH IgM | .23* | .15* | | |
| α-MSH IgG | .20* | .25* | .22* | |
| Age | .03 | .19* | -.05 | .04 |
| Socioeconomic status | -.03 | -.10* | .03 | -.02 |
| Body mass index | -.03 | .06 | -.01 | -.03 |
| Physicwal development | .02 | .09 | -.02 | .03 |
| C-reactive protein | .01 | .04 | .00 | .03 |
| HSV 1 IgG | .08 | .07 | .05 | .08 |
| HSV 2 IgG | .07 | .11* | .04 | .07 |
| EBV IgG | .04 | .05 | -.01 | .01 |
| HHV 6 IgG | -.01 | .08 | -.01 | .01 |
| Toxoplasma gondii IgG | .03 | .07 | .00 | .05 |
| Influenza A IgG | .12* | .12* | .03 | .13* |
| Influenza B IgG | .03 | -.09 | .09 | -.06 |

Spearman's rank correlations; * p<0.0008 (significant after Bonferroni correction for 64 tests)
Abbreviations: ACTH, adrenocorticotropic hormone; α-MSH, alpha-melanocyte stimulating hormone; Ig, immunoglobulin; HSV, herpes simplex virus; EBV, Epstein-Barr Virus; HHV, human herpesvirus

## Discussion

The aim of our study was to analyze the association between ACTH and α-MSH-reactive Ig levels and internalizing problems in adolescents. Our results show that levels of ACTH-reactive IgG are related to internalizing problems in adolescent girls from the general population. While there is high comorbidity between the two symptom scales 'Anxious/depressed' and 'Withdrawn/depressed', our finding present an indication that ACTH IgG are primarily involved in pathways regulating anxious and nervous behaviors, rather than in pathways that regulate withdrawn behaviors.

While little is known about the function and mechanism of action of ACTH IgG, the HPA axis is the most likely route by which ACTH IgG could affect emotion regulation and internalizing problems. In support of this mechanism, we have previously reported that higher total ACTH IgG levels were related to a higher cortisol recovery during a social stress test in girls from the same study sample [21], suggesting that total ACTH IgG may influence recovery after a strong reaction to a stressor.

The interaction between free and total ACTH IgG in predicting internalizing problems shows the complexity of this relationship. We observed two distinct situations. First, when levels of total ACTH IgG were high, the influence of free ACTH IgG was small. The level of internalizing problems was relatively high, regardless of levels of free ACTH IgG (Figure 1). Second, at low levels of total ACTH IgG, the level of free ACTH IgG determined the relationship of total ACTH IgG with internalizing

problems. Low total ACTH IgG in the presence of high free ACTH IgG were related to low internalizing problems, while low total ACTH IgG in the presence of low free ACTH IgG were related to high internalizing problems. One explanation for this delicate relationship may lie in different binding properties of free and total ACTH IgG. For example, 'free' (low affinity) ACTH IgG could transport ACTH and help to maintain an efficient cortisol response. In line with this, the level of internalizing problems was low when levels of free ACTH IgG were high and those of total ACTH IgG were low. On the other hand, high affinity ACTH IgG, the 'bound' fraction of total IgG, could neutralize ACTH and prevent it from eliciting a cortisol response, such as the blunted cortisol responses to stress seen in patients with depression or with panic disorder [14,30]. But the blunted response could also be a result of impaired transport of ACTH by ACTH IgG. A study of IgG reacting with ghrelin, a peptide hormone signaling hunger, has shown that increased affinity of IgG for ghrelin was associated with an enhanced orexigenic effect [31], suggesting that transportation was the mechanism. It is unknown which mechanism of action – neutralization or transportation – applies for ACTH IgG.

So far, little is known about factors influencing ACTH IgG production and their binding properties. One hypothesis is that exposure to microbial antigens that mimic melanocortin peptides could stimulate their production and change binding affinities [32,33]. For example, the amino acid sequence of ACTH shares similarities with influenza A and *Escherichia coli* (E.coli) proteins [32]. Interestingly, ACTH IgG levels in our study correlated positively with levels of influenza A-reactive IgG. Viral infections including influenza A have also been associated with a history of mood disorders [34]. Moreover, our recent study revealed that anti-influenza A IgG correlated with ghrelin-reactive IgG and IgM [35] suggesting that this viral infection may interfere with several peptide hormones involved in regulation of stress, mood and emotion. Furthermore, chronic supplementation of rats with commensal *E. coli* sex-dependently changed production and affinities of ACTH IgG and showed that the ACTH and α-MSH antibody response to *E.coli* are different [36]. The differences between melanocortin peptides may be related to the specific conformational epitopes present in bacterial proteins [33].

The relation between ACTH IgG and internalizing problems was only present in girls. Boys and girls also differed with regard to their levels of internalizing problems and levels of ACTH IgG; girls had higher levels of both. An explanation for the sex-specific association between internalizing problems and ACTH IgG may be that the significant relationship appears only at higher levels of both internalizing problems and ACTH IgG. Interestingly, higher levels of both ACTH IgG and a-MSH IgG are also present in female rats and have been related to sex-differences in gut bacterial composition [36].

Unlike previous studies [18,19], we found no relationship between α-MSH-reactive Ig levels and internalizing problems. Previous studies exploring associations between α-MSH autoAbs and behavior in humans were carried out in relatively small samples of

adults with autoimmune disease and healthy controls [19], or in patients with anorexia nervosa and bulimia (Fetissov 2005). The study of anorexia patients related α-MSH IgG and IgM to drive for thinness, social insecurity and impulse regulation [18]. These characteristics are not directly comparable to our measure of internalizing problems. Furthermore, unlike the other studies, we used a comparatively young and healthy study population, who may thus have had a relatively short history of exposure to stressors and antigens.



The strength of our study is its large sample size and its use of a population-based sample. A limitation is that we could not investigate the longitudinal dynamics of ACTH and α-MSH-reactive Ig levels. Second, total α-MSH IgG levels were not assessed, although, as we have shown for ACTH Ig levels, may give more insight into the dynamics of α-MSH Ig levels and internalizing problems. Third, as levels of internalizing problems were relatively mild in this general population sample, our findings do not necessarily extend to clinical samples.

## Conclusion and recommendations

Our study shows that ACTH-reactive IgG levels are related to internalizing problems in adolescent girls in the general population. Considering the role of the HPA axis in internalizing problems, it is plausible that ACTH-reactive IgG may influence internalizing problems via regulating stress physiology.

This is a novel and exciting field of research. Few studies have been performed so far. While our study gives promising results, we acknowledge the need for replication and further extension, not only in healthy adult populations, for example, but also in adolescents with clinical levels of anxiety and depression. More research is also needed to explain the sex differences. Future studies should aim to elucidate the longitudinal dynamics of ACTH and α-MSH-reactive Ig levels, should test whether changes in ACTH IgG levels are related to changes in internalizing problems and vice versa, and should investigate whether specific infections precede rises in ACTH IgG levels or changes in their affinity.

## References


1. Fergusson DM, Horwood LJ, Ridder EM, Beautrais AL (2005) Subthreshold depression in adolescence and mental health outcomes in adulthood. Arch Gen Psychiatry 62: 66-72.
2. Pine DS, Cohen P, Gurley D, Brook J, Ma Y (1998) The risk for early-adulthood anxiety and depressive disorders in adolescents with anxiety and depressive disorders. Arch Gen Psychiatry 55: 56-64.
3. Crum RM, Green KM, Storr CL, Chan YF, Ialongo N, et al. (2008) Depressed mood in childhood and subsequent alcohol use through adolescence and young adulthood. Arch Gen Psychiatry 65: 702-712.
4. Woodward LJ, Fergusson DM (2001) Life course outcomes of young people with anxiety disorders in adolescence. J Am Acad Child Adolesc Psychiatry 40: 1086-1093.
5. Jonas BS, Franks P, Ingram DD (1997) Are symptoms of anxiety and depression risk factors for hypertension? Longitudinal evidence from the National Health and Nutrition Examination Survey I Epidemiologic Follow-up Study. Arch Fam Med 6: 43-49.
6. Gantz I, Fong TM (2003) The melanocortin system. Am J Physiol Endocrinol Metab 284: E468-474.
7. Kokare DM, Dandekar MP, Singru PS, Gupta GL, Subhedar NK (2010) Involvement of alpha-MSH in the social isolation induced anxiety- and depression-like behaviors in rat. Neuropharmacology 58: 1009-1018.
8. Liu J, Garza JC, Truong HV, Henschel J, Zhang W, et al. (2007) The melanocortinergic pathway is rapidly recruited by emotional stress and contributes to stress-induced anorexia and anxiety-like behavior. Endocrinology 148: 5531-5540.
9. Lim BK, Huang KW, Grueter BA, Rothwell PE, Malenka RC (2012) Anhedonia requires MC4R-mediated synaptic adaptations in nucleus accumbens. Nature 487: 183-189.
10. Dhillo WS, Small CJ, Seal LJ, Kim MS, Stanley SA, et al. (2002) The hypothalamic melanocortin system stimulates the hypothalamo-pituitary-adrenal axis in vitro and in vivo in male rats. Neuroendocrinology 75: 209-216.
11. Lu XY, Barsh GS, Akil H, Watson SJ (2003) Interaction between alpha-melanocyte-stimulating hormone and corticotropin-releasing hormone in the regulation of feeding and hypothalamo-pituitary-adrenal responses. J Neurosci 23: 7863-7872.
12. Ludwig DS, Mountjoy KG, Tatro JB, Gillette JA, Frederich RC, et al. (1998) Melanin-concentrating hormone: a functional melanocortin antagonist in the hypothalamus. Am J Physiol 274: E627-633.
13. Pariante CM, Lightman SL (2008) The HPA axis in major depression: classical theories and new developments. Trends Neurosci 31: 464-468.
14. Petrowski K, Wintermann GB, Schaarschmidt M, Bornstein SR, Kirschbaum C (2013) Blunted salivary and plasma cortisol response in patients with panic disorder under psychosocial stress. Int J Psychophysiol 88: 35-39.
15. Lopez-Duran NL, Kovacs M, George CJ (2009) Hypothalamic-pituitary-adrenal axis dysregulation in depressed children and adolescents: a meta-analysis. Psychoneuroendocrinology 34: 1272-1283.
16. Booij SH, Bouma EM, de Jonge P, Ormel J, Oldehinkel AJ (2013) Chronicity of depressive problems and the cortisol response to psychosocial stress in adolescents: the TRAILS study. Psychoneuroendocrinology 38: 659-666.
17. Fetissov SO, Dechelotte P (2011) The new link between gut-brain axis and neuropsychiatric disorders. Curr Opin Clin Nutr Metab Care 14: 477-482.
18. Fetissov SO, Harro J, Jaanisk M, Jarv A, Podar I, et al. (2005) Autoantibodies against neuropeptides are associated with psychological traits in eating disorders. Proc Natl Acad Sci U S A 102: 14865-14870.

19. Karaiskos D, Mavragani CP, Sinno MH, Dechelotte P, Zintzaras E, et al. (2010) Psychopathological and personality features in primary Sjogren's syndrome--associations with autoantibodies to neuropeptides. Rheumatology (Oxford) 49: 1762-1769.

20. Sinno MH, Do Rego JC, Coeffier M, Bole-Feysot C, Ducrotte P, et al. (2009) Regulation of feeding and anxiety by alpha-MSH reactive autoantibodies. Psychoneuroendocrinology 34: 140-149.

21. Schaefer JM, Fetissov SO, Legrand R, Claeyssens S, Hoekstra PJ, et al. (2013) Corticotropin (ACTH)-reactive immunoglobulins in adolescents in relation to antisocial behavior and stress-induced cortisol response. The TRAILS study. Psychoneuroendocrinology.

22. Ormel J, Oldehinkel AJ, Sijtsema J, van Oort F, Raven D, et al. (2012) The TRacking Adolescents' Individual Lives Survey (TRAILS): design, current status, and selected findings. J Am Acad Child Adolesc Psychiatry 51: 1020-1036.

23. de Winter AF, Oldehinkel AJ, Veenstra R, Brunnekreef JA, Verhulst FC, et al. (2005) Evaluation of non-response bias in mental health determinants and outcomes in a large sample of pre-adolescents. European Journal of Epidemiology 20: 173-181.

24. Achenbach TM (1991) Manual for the Youth Self-Report and 1991 Profile. Department of Psychiatry, University of Vermont, Burlington, VT.

25. Fetissov SO (2011) Neuropeptide autoantibodies assay. Methods Mol Biol 789: 295-302.

26. Veenstra R, Lindenberg S, Oldehinkel AJ, De Winter AF, Ormel J (2006) Temperament, environment, and antisocial behavior in a population sample of preadolescent boys and girls. International Journal of Behavioral Development 30: 422-432.

27. Whicher JT, Ritchie RF, Johnson AM, Baudner S, Bienvenu J, et al. (1994) New international reference preparation for proteins in human serum (RPPHS). Clin Chem 40: 934-938.

28. Wang H, Yolken RH, Hoekstra PJ, Burger H, Klein HC (2011) Antibodies to infectious agents and the positive symptom dimension of subclinical psychosis: The TRAILS study. Schizophr Res 129: 47-51.

29. Williams JM, Dunlop LC (1999) Pubertal timing and self-reported delinquency among male adolescents. J Adolesc 22: 157-171.

30. Burke HM, Davis MC, Otte C, Mohr DC (2005) Depression and cortisol responses to psychological stress: a meta-analysis. Psychoneuroendocrinology 30: 846-856.

31. Takagi K, Legrand R, Asakawa A, Amitani H, Francois M, et al. (2013) Anti-ghrelin immunoglobulins modulate ghrelin stability and its orexigenic effect in obese mice and humans. Nat Commun 4: 2685.

32. Fetissov SO, Hamze Sinno M, Coeffier M, Bole-Feysot C, Ducrotte P, et al. (2008) Autoantibodies against appetite-regulating peptide hormones and neuropeptides: putative modulation by gut microflora. Nutrition 24: 348-359.

33. Tennoune N, Chan P, Breton J, Legrand R, Chabane YN, et al. (2014) Bacterial ClpB heat-shock protein, an antigen-mimetic of the anorexigenic peptide alpha-MSH, at the origin of eating disorders. Transl Psychiatry 4: e458.

34. Okusaga O, Yolken RH, Langenberg P, Lapidus M, Arling TA, et al. (2011) Association of seropositivity for influenza and coronaviruses with history of mood disorders and suicide attempts. J Affect Disord 130: 220-225.

35. Francois M, Schaefer JM, Bole-Feysot C, Dechelotte P, Verhulst FC, et al. (2015) Ghrelin-reactive immunoglobulins and anxiety, depression and stress-induced cortisol response in adolescents. The TRAILS study. Prog Neuropsychopharmacol Biol Psychiatry 59: 1-7.

36. Tennoune N, Legrand R, Ouelaa W, Breton J, Lucas N, et al. (2015) Sex-related effects of nutritional supplementation of Escherichia coli: Relevance to eating disorders. Nutrition 31: 498-507.

# chapter 4

## Ghrelin-reactive immunoglobulins and anxiety, depression and stress-induced cortisol response in adolescents. The TRAILS study.

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## Abstract

**Background:** Ghrelin, a hunger hormone, has been implicated in the regulation of stress-response, anxiety and depression. Ghrelin-reactive immunoglobulins (Ig) were recently identified in healthy and obese humans showing abilities to increase ghrelin's stability and orexigenic effects. Here we studied if ghrelin-reactive Ig are associated with anxiety and depression and with the stress-induced cortisol response in a general population of adolescents. Furthermore, to test the possible infectious origin of ghrelin-reactive Ig, their levels were compared with serum IgG against common viruses.

**Methods:** We measured ghrelin-reactive IgM, IgG and IgA in serum samples of 1199 adolescents from the Dutch TRAILS study and tested their associations with 1) anxiety and depression symptoms assessed with the Youth Self-Report, 2) stress-induced salivary cortisol levels and 3) IgG against Human Herpesvirus 1, 2, 4 and 6 and Influenza A and B viruses.

**Results:** Ghrelin-reactive IgM and IgG correlated positively with levels of antibodies against Influenza A virus. Ghrelin-reactive IgM correlated negatively with antibodies against Influenza B virus. Ghrelin-reactive IgM correlated positively with anxiety scores in girls and ghrelin-reactive IgG correlated with stress-induced cortisol secretion, but these associations were weak and not significant after correction for multiple testing.

**Conclusion:** These data indicate that production of ghrelin-reactive autoantibodies could be influenced by viral infections. Serum levels of ghrelin-reactive autoantibodies probably do not play a role in regulating anxiety, depression and the stress-response in adolescents from the general population.

## Introduction

Anxiety and depression in adolescents are a significant public health problem that may lead to serious and chronic mental disorders later in life. The present study was undertaken to better understand biological mechanisms that may underlie development of anxiety and depression in adolescents by looking for a possible link between these disorders and immunoglobulins (Ig)-reactive with ghrelin, a pleiotropic peptide hormone from the stomach, which has also been implicated in the regulation of internalizing behavior and physiological stress responses [1].



Ghrelin has been initially identified as a natural ligand of the growth hormone secretagogue receptor with a prominent effect to stimulate appetite [2], but later was also implicated in multiple physiological functions including regulation of stress-response, anxiety and mood [3]. Indeed, acute and chronic stress increased both ghrelin production and secretion in rodents and humans [3-9]. Increased ghrelin secretion has been suggested to alleviate the pathological consequences of stress, including stress-induced anxiety and depression [6]. In fact, when injected in rodents, ghrelin induced antidepressive-like behaviour [3,6,10,11] and under stress conditions, endogenous ghrelin seems to attenuate anxious-like behaviour and hypothalamic-pituitary-adrenal (HPA) axis activation [12]. However, several studies showed that ghrelin may also induce anxiogenic effects [4,13-17]. Thus, ghrelin seems to be a relevant target when studying anxiety, depression and altered stress response in adolescents but the underlying mechanisms are unknown.

Recently, ghrelin-reactive immunoglobulins (Ig) or autoantibodies were identified in humans and rodents [18,19]. It was found that ghrelin-reactive IgG protect ghrelin from degradation and may enhance its orexigenic effect, depending on its affinity for ghrelin, which was increased in obese humans and mice [20]. Thus, ghrelin-reactive Ig appear to play a role as modulators of ghrelin signaling, and hence, they could also be relevant to ghrelin's effects on anxiety, depression and stress-response. Because Ig production is regulated by multiple factors including specific antigens and non-specific activators of the humoral response by the immune system, serum levels of ghrelin-reactive Ig may reflect individual variability in ghrelin signaling. However, due to multiple chemical messengers implicated in the regulation of mood, emotion and stress, as well as typically subclinical manifestations of altered mood and emotion in adolescents, the possibility to reveal a significant link between ghrelin-reactive Ig levels and symptoms of anxiety and depression requires a relatively large number of study subjects. To address this issue, in the present study, ghrelin-reactive Ig were assayed in sera samples obtained from 1199 adolescents that took part in the Dutch Tracking adolescents' Individual Lives Survey (TRAILS) cohort study [21]. Although the antigenic origin of ghrelin-reactive Ig is uncertain, theoretically, according to the molecular mimicry concept [22], they can be stimulated by antigens homologous to ghrelin, which are present in some viruses and bacteria [18]. Because the occurrence of previous infections can be traced by the presence of specific anti-viral or anti-bacterial antibodies, it is possible to analyze if antibody levels can be associated with

serum levels of ghrelin-reactive Ig. Such associations may eventually point to a new molecular pathway linking infections and psychiatric disorders.

Thus, the aims of this study were to investigate possible associations between ghrelin-reactive Ig levels and 1) symptoms of anxiety and depression, 2) stress-induced cortisol levels and 3) serum IgG levels against common viruses including Herpes and Influenza.



## Subjects and Methods

### Study subjects

Data were collected from the TRAILS study, a large prospective cohort study taking place in the Netherlands which examines the development of mental and physical health from pre-adolescence into adulthood [21,23]. The study was approved by the National Dutch Medical Ethics Committee. Thus far, five assessment waves of TRAILS have been completed. For this study we used data from the third assessment, running from September 2005 to December 2007 (T3). After intensive recruitment efforts, a total of 2230 children (response rate 76.0%, age range 10-12 years, 50.8% girls) were included at T1, of whom 1816 participated in T3 (response rate 81.4%, age range 15-18 years, 52.3% girls). Measurements consisted of validated questionnaires, interviews and biological measures. During T3, blood serum was collected from 1230 participants, and 715 agreed to perform a series of laboratory tasks (experimental session), including the Groningen Social Stress (GSST), a modified version of the Trier Social Stress Test [24]. From the first 1230 blood samples collected during T3, 1199 were available for this study. From the 715 adolescents that agreed to perform the experimental session, 560 blood samples were available. We excluded adolescents using corticosteroids (n=8) and girls using oral contraceptives (OC, n=98) from the cortisol analyses.

### Experimental session and the social stress test

During the experimental session, participants' psycho-physiological responses to a variety of challenging conditions were recorded. These conditions included a spatial orienting task, a gambling task, a startle reflex task, and a social stress test. The session took place on weekends, in soundproof rooms with blinded windows. The total session started between 08:00 and 09:30 or between 12:00 and 14:30 and lasted about 3 hours and 15 minutes. Although free salivary cortisol levels may be higher in the morning due to the circadian rhythm of cortisol production, morning and afternoon cortisol responses to social stress have been reported to be comparable [25]. Participants were asked to refrain from smoking and from having coffee, milk, chocolate, and other sugar-containing foods in the 2h before the test. The protocol was approved by the Central Committee on Research Involving Human Subjects (CCMO). All participants gave informed consent.

The GSST was the last challenge of the experimental session. The test involved a standardized protocol aiming to induce a moderate performance-related social stress. Adolescence is characterized by major biological, psychological and social challenges that are known to be effective activators of various psychological stress systems, particularly in combination with uncontrollability. Thus, the social stress test consisted of two parts. Participants were first instructed to prepare a speech of 6 minutes about themselves and their lives and deliver this speech in front of a video camera. They were told that their videotaped performance would be judged on content of speech as well as on use of voice and posture and that it would be rank-ordered by a panel of peers after the experiment. The participants had to speak continuously for the whole period of 6 min. The test assistant watched the performance critically, without showing empathy or encouragement. The speech was followed by a 3-min interlude in which the participants were not allowed to speak. Subsequently, during the second part of the test, participants were asked to perform mental arithmetic. They were instructed to repeatedly subtract the number 17 repeatedly, starting with 13,27

This difficult task was meant to induce a sense of uncontrollability that was further provoked by negative feedback from the test assistant. This mental arithmetic task lasted 6 minutes, followed by period of silence of 3 minutes, after which the participants were debriefed about the experiment.

## Cortisol

The HPA axis responses toward the GSST was assessed by four saliva cortisol samples: (1) before the test, representing pre-test cortisol levels, (2) directly after the test, reflecting HPA axis responses during speech, (3) 20 minutes after the test, reflecting levels at the end of the test, and (4) 40 minutes after the end of the GSST, representing post-test cortisol levels. Considering the normal delay (20-25 minutes) in peak cortisol response to experimental stressors[26], all measures reflected cortisol levels about 20 min earlier.

Saliva was collected using the Salivette sampling device (Sarstedt, Numbrecht, Germany) and samples were stored at -20°C until analysis. Cortisol concentrations were measured in duplicates from 100 µl saliva using an in-house radioimmunoassay applying a polyclonal rabbit cortisol antibody and 1, 2, 6, 7 $^{3}$H cortisol (Amersham, Arlington Heights, IL) as tracer. After incubation for 30 min at 60°C, the bound and free fractions were separated using active charcoal. The intra-assay coefficient of variation was 8.2% for concentrations of 1.5 nM, 4.1% for concentrations of 15 nM, and 5.4% for concentration of 30 nM. The inter-assay coefficients of variation were, respectively, 12.6%, 5.6% and 6.0%. The detection limit was 0.9 nM.

The amount of cortisol released during the GSST (incremental area under the curve with respect to increase, AUCincr) was calculated over the four measures taken during the GSST using the trapezoid formula [27]. Recovery was calculated by subtracting post-test values from the peak.

## Anxiety and depression

Internalizing problems were assessed with the Youth Self-Report (YSR), which is one of the most commonly used self-report questionnaires in current child and adolescent psychiatric research [28]. The YSR contains 112 items on behavioural and emotional problems in the past six months. Participants could rate each item on a three-point scale (0= not true, 1= somewhat or sometimes true, 2= very true or often true). For the present study, we used two scales of the YSR: 'Anxious/depressed' (13 items, Chronbach's $\alpha$ =0.84; e.g., 'I cry a lot', 'I am afraid of going to school', 'I am nervous or tense', 'I worry a lot') and 'Withdrawn/ depressed' (8 items, Chronbach's $\alpha$ =0.74, e.g., 'There is very little that I enjoy', 'I would rather be alone than with others', 'I am unhappy, sad or depressed').

## Ghrelin-reactive immunoglobulins

Ghrelin Ig levels were measured in serum using an enzyme-linked immunosorbent assay (ELISA) [29]. Briefly, 96-well plates (Nunc Immunoplate, Rochester, NY) were coated with 2 mg/ml of human ghrelin (Bachem, Bubendorf, Switzerland) in 0.5 M $Na_2CO_3$, 0.5 M $NaHCO_3$, pH 9.6, and incubated for 72 h at 4°C. Plates were washed using phosphate buffered saline (PBS, 0.05% Tween20, pH 7.4). Serum was diluted 1:100 in either physiological buffer (PBS, 0.02% sodium azide, pH 7.4) to measure free ghrelin IgA, IgM and IgG or in 3 M NaCl, 1.5 M glycine (pH 8.9), creating a dissociation condition to measure total ghrelin IgG levels. After incubation at 4°C overnight, plates were washed and incubated for 3 h with alkaline phosphatase-conjugated secondary antibody (anti-human IgA, IgM or IgG; Jackson ImmunoResearch, West Grove, PA). The reaction was developed for 40 minutes with 150 µl p-Nitrophenyl phosphatase substrate solution (Sigma, St Louis, MO) at 1 mg/ml in 0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2$-$6H_20$, pH 9.

100 µl NaOH (2M) was added to stop the reaction and optical density (OD) was measured at 405 nm in an ELISA plate reader. The intra-assay coefficients of variation were 4.62% for class A of ghrelin-reactive Ig, 3.13% for class M of ghrelin-reactive Ig, 3.26% for free ghrelin-reactive IgG and 3.94% for total ghrelin-reactive IgG. The inter-assay coefficients of variation were, respectively, 4.6%, 9.5%, 6.8% and 8.1%.

## Antibodies against infectious agents

The concentrations of IgG serum antibodies against Influenza A and B, human herpesvirus 6 (HHV-6), herpes simplex virus 1 and 2 (HSV-1 and HSV-2) and Epstein-Barr Virus (EBV) were measured using solid-enzyme immunoassay methods, as described previously [30]. The assays were performed by the reaction of diluted aliquots of adolescent samples to antigens immobilized onto a solid-phase surface, with the subsequent quantification of IgG antibodies by reaction of bound antibodies with enzyme-labeled anti-human IgG and enzyme substrate. The optical

density of the ensuing enzyme-substrate reaction was quantified by means of spectrophotometric instrumentation.

## Covariates

We considered the following variables as covariates: sex, age, physical development, socioeconomic status, body mass index and smoking. *Physical development* (PD) was assessed by self-report using the physical development scale [31]. Z-Scores for PD were calculated for boys and girls separately (missing n=41). *Socioeconomic status (SES)* was calculated as the Z-score of the mean of educational level (father/mother), occupational level (father/mother), and family income at T1 (missing n= 13) [32]. *Body mass index (BMI)* was calculated as weight in kg divided by height in m² (missing n=29). *Smoking* behaviour was assessed by questions on past and current smoking behaviour in a questionnaire which was filled out at school, on average 3.07 months (SD=5.12) before the experimental session. We distinguished between non-habitual smokers and habitual smokers (≥ 1 cigarette/week) (missing n=9).

## Statistical analyses

Descriptives for age, PD, BMI, SES, anxiety and depression symptom scores, HPA axis activity, and ghrelin-reactive Ig levels were computed. Independent t-tests were used to compare boys and girls, OC-users and non-OC-users (SPSS version 21.0). We used linear regression models to study the relationship between ghrelin-reactive Ig and anxiety and depression, between ghrelin-reactive Ig and cortisol response, and finally between ghrelin-reactive Ig and IgG against infectious agents. We tested the influence of potential covariates and included them in the models when they changed the regression coefficients of the independent variables. Linear regression analyses were conducted with ghrelin-reactive Ig as independent and anxiety and depression as dependent variables, with sex and age as covariates. We tested whether sex moderated the relationship between ghrelin-reactive Ig and anxiety or depression by adding an interaction term (sex x ghrelin-reactive Ig) to the model. Linear regression analyses were conducted with ghrelin-reactive Ig as independent and cortisol measures (AUCincr or Recovery) as dependant variables, with sex, age, SES, BMI, PD and smoking as covariates. Cortisol measures were transformed using natural logarithm before analyses. As in previous analyses, we tested for a moderating effect of sex on the relationship between Ig levels and cortisol measures. Linear regression analyses were also done with ghrelin-reactive Ig as independent variable, and IgG antibodies to viruses as dependant variable. Due to the large number of comparisons in our study we adjusted analyses for multiple testing using the Bonferroni correction. We applied this correction per research question that was tested in this study. The new p-values regarded as significant were $p<0.004$ for analyses of the associations between ghrelin-reactive Ig and anxiety or depression (Bonferroni correction for 12 tests), and $p<0.002$ for analyses of associations between ghrelin-reactive Ig and stress

responses or antibodies against infectious agents (Bonferroni correction for 24 tests). Missing values were excluded list-wise.

## Results



### Subjects characteristics

A description of the study subjects and variables are shown in Table
Girls had higher anxiety and depression scores than boys. Girls had significantly higher mean levels of free and total ghrelin-reactive IgG as well as higher levels of ghrelin-reactive IgM than boys. There was no difference in levels of ghrelin-reactive IgA between boys and girls. Finally, boys had higher cortisol response and recovery after the GSST than girls. Cortisol response was blunted in girls using OC as shown previously [24].

**Table 1**

| | Boys | | | Girls | | |
|---|---|---|---|---|---|---|
| | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** |
| Age (years) | 563 | 16.19 | 0.64 | 636 | 16.21 | 0.68 |
| Physical development | 544 | 0.02 | 0.96 | 613 | 0.01 | 0.99 |
| BMI (kg/m²) | 552 | 20.85*** | 3.15 | 618 | 21.62 | 3.06 |
| Socioeconomic status | 556 | 0.12 | 0.79 | 630 | 0.10 | 0.76 |
| Withdrawn/Depressed (YSR) | 547 | 0.32*** | 0.30 | 621 | 0.42 | 0.33 |
| Anxious/Depressed (YSR) | 547 | 0.19*** | 0.21 | 621 | 0.38 | 0.32 |
| Ghrelin-reactive Ig (OD) | | | | | | |
| IgA | 563 | 0.08 | 0.08 | 636 | 0.07 | 0.08 |
| IgM | 563 | 0.55*** | 0.32 | 636 | 0.68 | 0.37 |
| Free IgG | 562 | 0.63* | 0.37 | 635 | 0.68 | 0.40 |
| Total IgG | 561 | 1.69*** | 0.59 | 635 | 1.83 | 0.58 |
| Salivary cortisol after stress | | | | | | |
| AUCincr | 261 | 83.13** | 106.26 | 178 (no OC) | 72.78[a] | 118.31 |
| | | | | 95 (OC) | 20.48 | 43.79 |
| Recovery (nmol/l) | 254 | 1.74*** | 2.20 | 259 | 0.94[b] | 2.66 |

Characteristics of the study samples. YSR, Youth Self Report; OD, optical density; Ig, immunoglobulins; HPA, hypothalamic-pituitary-adrenal; AUCincr, area under the curve of salivary cortisol release during social stress test with respect to starting value; Recovery, difference between stress-induced peak of cortisol and its post-stress value; OC, oral contraceptives; SD, standard deviation. [a]p<0.001 difference between girls taking OC and girls not taking OC. [b]No difference between girls taking OC and girls not taking OC. *p<0.05 **p<0,01 ***p<0,001.

### Ghrelin-reactive Ig and anxiety/depression

We did not find any significant associations between ghrelin-reactive Ig and anxiety or depression scores in the total sample of adolescents (Table 2 and 3). Interactions with sex were not significant, however, due to the differences in Ig levels and in anxiety and

depression scores, we decided to analyse boys and girls separately. In girls, ghrelin-reactive IgM was positively associated with anxiety (Table 2), but this association did not persist after correction for multiple testing. Other classes of ghrelin-reactive Ig did not show significant associations with anxiety (Table 2). No significant associations between ghrelin-reactive Ig and depression were found (Table 3).



**Table 2:** Linear regression coefficients (β) between plasma levels of ghrelin-reactive immunoglobulins (Ig) and anxiety score (YSR) in the whole cohort of adolescents and in boys and girls separately.

| Ghrelin-reactive Ig | Boys and girls | | | Boys | | | Girls | | |
|---|---|---|---|---|---|---|---|---|---|
| | β | 95% CI | p | β | 95% CI | p | β | 95% CI | p |
| IgA | 0,09 | -0,10; 0,29 | 0,35 | 0,01 | -0,20; 0,22 | 0,95 | 0,19 | -0,14; 0,52 | 0,27 |
| IgM | 0,04 | -0,01; 0,08 | 0,10 | -0,01 | -0,06; 0,05 | 0,79 | 0,07 | 0,00; 0,14 | 0,048 |
| Free IgG | 0,03 | -0,01; 0,07 | 0,16 | 0,00 | -0,04; 0,05 | 0,88 | 0,05 | -0,02; 0,11 | 0,14 |
| Total IgG | -0,01 | -0,04; 0,02 | 0,59 | 0,01 | -0,03; 0,04 | 0,73 | -0,02 | -0,06; 0,03 | 0,39 |

Covariate in the models: age, sex (total sample). CI, confidence interval.

**Table 3:** Linear regression coefficients (β) between plasma levels of ghrelin-reactive immunoglobulins (Ig) and depression score (YSR) in the whole cohort of adolescents and in boys and girls separately.

| Ghrelin-reactive Ig | Boys and girls | | | Boys | | | Girls | | |
|---|---|---|---|---|---|---|---|---|---|
| | β | 95% CI | p | β | 95% CI | p | β | 95% CI | p |
| IgA | 0,06 | -0,17; 0,29 | 0,61 | -0,14 | -0,44; 0,15 | 0,35 | 0,28 | -0,07; 0,62 | 0,11 |
| IgM | 0,05 | -0,00; 0,10 | 0,06 | 0,07 | -0,01; 0,15 | 0,08 | 0,04 | -0,03; 0,11 | 0,29 |
| Free IgG | 0,04 | -0,01; 0,08 | 0,14 | 0,03 | -0,04; 0,10 | 0,40 | 0,04 | -0,03; 0,11 | 0,23 |
| Total IgG | 0,00 | -0,03; 0,03 | 0,89 | -0,01 | -0,05; 0,04 | 0,79 | 0,00 | -0,04; 0,05 | 0,96 |

Covariate in the models: age, sex (total sample). CI, confidence interval.

## Ghrelin-reactive Ig and stress-induced cortisol response

Levels of total ghrelin-reactive IgG were negatively associated with cortisol response during the social stress test in the total sample (Table 4). No association with recovery was found (Table 4). Interactions with sex were not significant. However, due to sex differences in ghrelin-reactive Ig levels and the stress-induced cortisol response, we decided to stratify the sample. In girls, association between total ghrelin-reactive IgG and the cortisol response was stronger than in boys (Table 4), but none of the associations was significant after correction for multiple testing.

## Ghrelin-reactive Ig and anti-viral antibodies

Ghrelin-reactive IgM and IgG were found positively associated with IgG antibodies against influenza A in the total sample of adolescents (Table 5). Ghrelin-reactive IgM

were negatively associated with antibodies against influenza B. These associations persisted even after correction for multiple testing. Ghrelin-reactive IgM were also positively associated with antibodies against HSV-2, and IgA correlated negatively with Influenza B antibodies, but these associations were weak and not significant after correction for multiple testing.



**Table 4:** Linear regression coefficients (β) between plasma levels of ghrelin-reactive immunoglobulins (Ig) and HPA axis activity during social stress, including cortisol release and recovery in the whole cohort of adolescents and in boys and girls separately.

| Ghrelin- | AUCincr | | | Recovery | | |
|---|---|---|---|---|---|---|
| reactive Ig | β | 95% CI | p | β | 95% CI | p |
| Boys and Girls | | | | | | |
| IgA | 3,99 | -1,38; 9,36 | 0,14 | 1,15 | -1,90; 4,19 | 0,46 |
| IgM | -0,42 | -1,56; 0,73 | 0,47 | -0,60 | -1,23; 0,04 | 0,07 |
| Free IgG | 0,28 | -0,61; 1,16 | 0,53 | 0,32 | -0,18; 0,83 | 0,21 |
| Total IgG | -0,79 | -1,51; -0,06 | 0,03 | -0,20 | -0,65; 0,25 | 0,38 |
| Boys | | | | | | |
| IgA | 3,49 | -5,04; 12,02 | 0,42 | 2,66 | -2,28; 7,60 | 0,28 |
| IgM | -1,09 | -3,15; 0,98 | 0,30 | -0,68 | -1,75; 0,39 | 0,21 |
| Free IgG | 0,13 | -1,04; 1,30 | 0,83 | 0,50 | -0,21; 1,21 | 0,17 |
| Total IgG | -0.36 | -1.33 ; 0.60 | 0.45 | -0.18 | -0.77 ; 0.41 | 0.55 |
| Girls | | | | | | |
| IgA | 4,23 | -3,49; 11,94 | 0,28 | 1,11 | -3,13; 5,34 | 0,60 |
| IgM | -0,004 | -1,52; 1,51 | 1,00 | -0,39 | -1,24; 0,46 | 0,35 |
| Free IgG | 0,24 | -1,24; 1,72 | 0,74 | 0,28 | -0,49; 1,06 | 0,46 |
| Total IgG | -1.36 | -2.52 ; -0.20 | 0.02 | -0,10 | -0.92 ; 0.73 | 0,82 |

Covariates in the model: sex (total sample), age, physical development, socioeconomic status, BMI, smoking. Girls taking OC excluded. AUCincr, area under the curve during social stress test with respect to starting value; Recovery, cortisol recovery after social stress test; CI, confidence interval; OC, oral contraceptives.

**Table 5:** Linear regression coefficient (β) between ghrelin-reactive immunoglobulins (Ig) and IgG antibody against several common viruses in adolescents.

| | IgA | | IgM | | Free IgG | | Total IgG | |
|---|---|---|---|---|---|---|---|---|
| Plasma IgG against | β | p | β | p | β | p | β | p |
| Herpes simplex virus type 1 (HSV-1) | -0,03 | 0,94 | 0,003 | 0,97 | 0,11 | 0,13 | 0,04 | 0,36 |
| Herpes simplex virus type 2 (HSV-2) | 0,01 | 0,90 | 0,04 | 0,047 | 0,01 | 0,76 | -0,01 | 0,45 |
| Epstein Barr Virus (HHV-4) | 0,20 | 0,32 | -0,09 | 0,06 | 0,04 | 0,36 | 0,004 | 0,89 |
| Human Herpesvirus 6 (HHV-6) | 0,07 | 0,94 | 0,34 | 0,13 | 0,02 | 0,92 | 0,01 | 0,97 |
| Influenza A | 0,24 | 0,30 | 0,24 | <0,001 | 0,19 | <0,001 | 0,07 | 0,04 |
| Influenza B | -0,33 | 0,04 | -0,15 | <0,001 | -0,002 | 0,95 | 0,002 | 0,92 |

Covariates in the model: sex, age, BMI. Bold values: p<0.002 (new p-value regarded as significant after application of Bonferroni correction for multiple testing).

## Discussion

Our study showed that serum levels of ghrelin-reactive Ig correlated with antibody levels against Influenza A virus and Influenza B virus. Our study further suggests that serum levels of ghrelin-reactive IgM might be positively associated with anxiety in girls and that increased levels of total ghrelin-reactive IgG might be associated with a lower activation of the HPA axis during a social stress test in adolescents. However, these correlations were weak and are most probably chance findings.



This is the first study exploring the serum levels of ghrelin-reactive Ig in a large representative sample of adolescents from the general population. Previously, presence of ghrelin-reactive Ig in humans was shown in healthy, obese or anorectic adult females [18-20]. Thus, this study confirms the ubiquitous presence of ghrelin-reactive Ig in adolescents of both genders, supporting a role of such Ig in humans as modulators of ghrelin signaling. It is of importance to note that this study was exploratory in methodology, and therefore, results should be interpreted cautiously, especially as a high number of tests were carried out, which increases the risk of chance findings.

How may these results help to clarify mechanisms underlying development of anxiety and depression in adolescents? Previous data has established that ghrelin-reactive IgG play a protective role by preventing degradation of ghrelin by plasma enzymes, i.e. its deacylation and loss of biological activity [20]. Therefore, one may assume that increased serum levels of ghrelin-reactive IgG would improve ghrelin's stability, and hence, will increase its effects on anxiety, depression and stress-response [6]. Although the functional role of ghrelin-reactive IgM or IgA has not been previously established, it is likely that they may also contribute to ghrelin's protection from degradation. Furthermore, detection of the IgM class may signify a recent exposure to ghrelin-like antigens, while IgA may point to an intra-luminal antigenic source, typically the gut content, including food and microbial antigens, but also respiratory tract antigens.

Previous data implicated ghrelin in both anxiogenic and anxiolytic responses [6], and while the mechanisms underlying such differences are presently unclear, the following discussion supports anxiogenic ghrelin actions based on our results which tentatively suggest weak positive associations between ghrelin-reactive IgM and anxiety.

Accordingly, increased levels of ghrelin-reactive IgM may contribute to increased anxiety in girls by promoting ghrelin's stability and anxiogenic effects in physiological conditions [4,13-17]. In agreement with this possibility, a recent study reported increased plasma levels of ghrelin in subjects with depression which was normalized after treatment [33]. It should be, however, noted that the correlation between ghrelin-reactive IgM and anxiety were weak and not significant after correction for multiple testing. It is therefore likely that this result is a chance finding, considering that these analyses were largely exploratory and that the probability of finding a significant result when there is truly no effect is 0.71 when 24 tests are carried out or 0.46 when

12 tests are done [34]. Hence, replication and mechanistic studies are warranted. Furthermore, it indicates that probably there is no functional role of ghrelin-reactive Ig in internalizing problems in this general population of adolescents. Alternatively, ghrelin and ghrelin-reactive Ig, may provide a relatively modest contribution to the regulation of mood and emotion in healthy adolescents, among many other hormones and messengers [35,36], but might be confined to specific patient populations (e.g. patients with eating disorders, anxiety disorders or depression). Another possibility is that a relationship between ghrelin-reactive Ig and anxiety and depression could be more specific for acute stress-induced anxiety and depression, similarly to what has been found for ghrelin (Spencer, Xu, et al. 2012), but this relationship was not tested in our study.

Ghrelin has been shown to activate the HPA axis [37,38]. Our study suggests that total but not free ghrelin-reactive IgG might correlate negatively with cortisol release during social stress. Because the IgG class undergoes affinity maturation, which is minimal for the IgM and IgA classes, IgG become immunogenic and are naturally neutralized by anti-idiotypic antibodies [39,40]. Therefore, an increase in ghrelin-reactive total IgG levels and at the same time decrease of free IgG levels may signify a decrease of their functional abilities to protect ghrelin, which would diminish ghrelin's ability to activate the HPA axis to cope with stress and anxiety. It further indicates a history of affinity maturation due to stimulation by ghrelin-like antigens. These associations appear antigen specific, because a previous study using the same sample of adolescents, found a negative association between AUCincr of cortisol and free but not total corticotropin-reactive IgG [41]. Insufficient acute stress-induced activation of cortisol secretion was previously shown in subjects with mild and moderate depression [42], and ghrelin-reactive free IgG may play a role in improving ghrelin's protective role during stress [6]. We could not confirm this hypothesis in our study, as there was no significant associations between ghrelin-reactive free IgG and cortisol responses. But, it would be interesting to study such associations in patients with depression.

The ubiquitous presence of ghrelin-reactive Ig indicates that production of such autoantibodies could be potentially triggered by ghrelin-like antigens. To explore the possibility of an infectious origin of ghrelin-reactive Ig, we compared their serum levels with antibodies against common viruses including Herpes virus and Influenza. We found that ghrelin-reactive IgM and IgG correlated positively with antibodies against Influenza A, suggesting that this infection may trigger their production. Based on these data, we looked for sequence homologies between ghrelin and Influenza A virus using the protein sequence alignment tool from the NCBI web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). We found that the Influenza A PB1-F2 protein displayed the highest score of homology with ghrelin, including five consecutive amino acids [43]. The PB1-F2 protein is an important virulence factor [44], inducing anti-PB1-F2 antibody response in Influenza-infected humans and mice [45], and its potential ability to interfere with the ghrelin system is intriguing. The presence of sequence

homology, however, does not necessarily signify functional molecular mimicry, and should be experimentally validated [46]. In contrast to Influenza A, correlations between ghrelin-reactive Ig and antibodies against Influenza B virus were negative and were present for the IgM, but not IgG classes. One explanation of such difference may implicate the presence of potential ghrelin-like antigens in influenza A but not influenza B viruses, e.g. PB1-F2 protein [47]. Furthermore, the tentative correlation between Influenza B and ghrelin-reactive IgA suggests that they could be stimulated by a naturally exposed Influenza B virus to the respiratory or digestive tract. However, low detection levels of ghrelin-reactive IgA in general do not support a functional role of such Ig in ghrelin signaling. The tentative correlation between ghrelin-reactive IgM and anti-HSV-2 antibodies would be in line with a study that found increased anxiety shortly after HSV-2 virus reactivation [48]. However, this correlation was weak, and hence, might also be a chance finding.



It should be noted that although a modulatory role of ghrelin reactive Ig in ghrelin signaling exists [20], correlations between behavioral traits and serum levels of these autoantibodies may not necessarily reflect their direct involvement in the mechanisms of anxiety and depression. An alternative explanation of such correlations would be that ghrelin-reactive Ig are biomarkers of ghrelin-like microbial antigens that may modulate ghrelin receptors directly. Such a mechanism, involving an α-melanocyte-stimulating hormone (MSH)-like bacterial antigen and anti-α-MSH autoantibodies was recently described in eating disorders [46]. While our study does not provide evidence for the direct or indirect involvement of ghrelin-reactive Ig in anxiety, depression and stress response in adolescents from the general population, it is possible that such relationships might exist in specific populations of adolescents, e.g. patients with eating disorders, anxiety disorders or depression. This needs to be further tested. Significant correlations with anti-Influenza antibodies could indicate a molecular link between viral infections and regulation of stress, mood and emotion that involves ghrelin signaling. This hypothesis should be further investigated. It would be in agreement with previous data showing increased anxiety and depression in subjects after influenza A infections [49,50] and with numerous studies supporting the involvement of autoantibodies in anxiety and depression [51] and the infectious origin of psychiatric disorders [52].

## Conclusion

In conclusion, we showed that serum levels of ghrelin-reactive Ig are associated with anti-Influenza antibody levels. The association between ghrelin-reactive Ig and symptoms of anxiety and stress-reactivity in girls were weak and most likely due to chance, replication studies are warranted. Future studies will be necessary to prove whether Influenza infections cause production of ghrelin-reactive Ig, and whether they in turn could influence anxiety and the stress response. If correlations between these environmental, molecular and behavioural factors are found, this would support a mechanistic link that may help to understand the development of anxiety and

depression in adolescents. Further studies, for example in patient groups, could be useful to test this link and the utility of targeting ghrelin-reactive Ig production as a new strategy for prevention and treatment of anxiety and depression in adolescents.

## References


1. Delporte C (2013) Structure and Physiological Actions of Ghrelin. Scientifica 2013: 25.
2. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, et al. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402: 656-660.
3. Chuang JC, Zigman JM (2010) Ghrelin's Roles in Stress, Mood, and Anxiety Regulation. Int J Pept 2010.
4. Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, et al. (2001) A role of ghrelin in neuroendocrine and behavioral responses to stress in mice. Neuroendocrinology 74: 143-147.
5. Kristenssson E, Sundqvist M, Astin M, Kjerling M, Mattsson H, et al. (2006) Acute psychological stress raises plasma ghrelin in the rat. Regul Pept 134: 114-117.
6. Lutter M, Sakata I, Osborne-Lawrence S, Rovinsky SA, Anderson JG, et al. (2008) The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. Nat Neurosci 11: 752-753.
7. Ochi M, Tominaga K, Tanaka F, Tanigawa T, Shiba M, et al. (2008) Effect of chronic stress on gastric emptying and plasma ghrelin levels in rats. Life Sci 82: 862-868.
8. Rouach V, Bloch M, Rosenberg N, Gilad S, Limor R, et al. (2007) The acute ghrelin response to a psychological stress challenge does not predict the post-stress urge to eat. Psychoneuroendocrinology 32: 693-702.
9. Zheng J, Dobner A, Babygirija R, Ludwig K, Takahashi T (2009) Effects of repeated restraint stress on gastric motility in rats. Am J Physiol Regul Integr Comp Physiol 296: R1358-1365.
10. Carlini VP, Machado DG, Buteler F, Ghersi M, Ponzio MF, et al. (2012) Acute ghrelin administration reverses depressive-like behavior induced by bilateral olfactory bulbectomy in mice. Peptides 35: 160-165.
11. Kanehisa M, Akiyoshi J, Kitaichi T, Matsushita H, Tanaka E, et al. (2006) Administration of antisense DNA for ghrelin causes an antidepressant and anxiolytic response in rats. Prog Neuropsychopharmacol Biol Psychiatry 30: 1403-1407.
12. Spencer SJ, Xu L, Clarke MA, Lemus M, Reichenbach A, et al. (2012) Ghrelin regulates the hypothalamic-pituitary-adrenal axis and restricts anxiety after acute stress. Biological Psychiatry 72: 457-465.
13. Carlini VP, Monzon ME, Varas MM, Cragnolini AB, Schioth HB, et al. (2002) Ghrelin increases anxiety-like behavior and memory retention in rats. Biochem Biophys Res Commun 299: 739-743.
14. Carlini VP, Varas MM, Cragnolini AB, Schioth HB, Scimonelli TN, et al. (2004) Differential role of the hippocampus, amygdala, and dorsal raphe nucleus in regulating feeding, memory, and anxiety-like behavioral responses to ghrelin. Biochem Biophys Res Commun 313: 635-641.
15. Currie PJ, Khelemsky R, Rigsbee EM, Dono LM, Coiro CD, et al. (2012) Ghrelin is an orexigenic peptide and elicits anxiety-like behaviors following administration into discrete regions of the hypothalamus. Behav Brain Res 226: 96-105.
16. Currie PJ, Schuette LM, Wauson SE, Voss WN, Angeles MJ (2014) Activation of urocortin 1 and ghrelin signaling in the basolateral amygdala induces anxiogenesis. Neuroreport 25: 60-64.
17. Hansson C, Haage D, Taube M, Egecioglu E, Salome N, et al. (2011) Central administration of ghrelin alters emotional responses in rats: behavioural, electrophysiological and molecular evidence. Neuroscience 180: 201-211.

18. Fetissov SO, Hamze Sinno M, Coëffier M, Bole-Feysot C, Ducrotté P, et al. (2008) Autoantibodies against appetite-regulating peptide hormones and neuropeptides: putative modulation by gut microflora. Nutrition 24: 348-359.

19. Terashi M, Asakawa A, Harada T, Ushikai M, Coquerel Q, et al. (2011) Ghrelin reactive autoantibodies in restrictive anorexia nervosa. Nutrition 27: 407-413.

20. Takagi K, Legrand R, Asakawa A, Amitani H, François M, et al. (2013) Anti-ghrelin immunoglobulins modulate ghrelin stability and its orexigenic effect in obese mice and humans. Nat Commun 4:2685.

21. Huisman M, Oldehinkel AJ, de Winter A, Minderaa RB, de Bildt A, et al. (2008) Cohort profile: the Dutch 'TRacking Adolescents' Individual Lives' Survey'; TRAILS. Int J Epidemiol 37: 1227-1235.

22. Oldstone MB (2005) Molecular mimicry, microbial infection, and autoimmune disease: evolution of the concept. Curr Top Microbiol Immunol 296: 1-17.

23. de Winter AF, Oldehinkel AJ, Veenstra R, Brunnekreef JA, Verhulst FC, et al. (2005) Evaluation of non-response bias in mental health determinants and outcomes in a large sample of pre-adolescents. Eur J Epidemiol 20: 173-181.

24. Bouma EM, Riese H, Ormel J, Verhulst FC, Oldehinkel AJ (2009) Adolescents' cortisol responses to awakening and social stress; effects of gender, menstrual phase and oral contraceptives. The TRAILS study. Psychoneuroendocrinology 34: 884-893.

25. Kudielka BM, Schommer NC, Hellhammer DH, Kirschbaum C (2004) Acute HPA axis responses, heart rate, and mood changes to psychosocial stress (TSST) in humans at different times of day. Psychoneuroendocrinology 29: 983-992.

26. Kirschbaum C, Read GF, Hellhammer DH (1992) Assessment of Hormones and Drugs in Saliva in Biobehavioral Research. Hogrefe & Huber.

27. Janssens KA, Oldehinkel AJ, Verhulst FC, Hunfeld JA, Ormel J, et al. (2012) Symptom-specific associations between low cortisol responses and functional somatic symptoms: the TRAILS study. Psychoneuroendocrinology 37: 332-340.

28. Achenbach TM, Dumenci L, Rescorla LA (2003) DSM-oriented and empirically based approaches to constructing scales from the same item pools. J Clin Child Adolesc Psychol 32: 328-340.

29. Fetissov SO (2011) Neuropeptide autoantibodies assay. Methods Mol Biol 789: 295-302.

30. Wang H, Yolken RH, Hoekstra PJ, Burger H, Klein HC (2011) Antibodies to infectious agents and the positive symptom dimension of subclinical psychosis: The TRAILS study. Schizophrenia Research 129: 47-51.

31. Williams JM, Dunlop LC (1999) Pubertal timing and self-reported delinquency among male adolescents. J Adolesc 22: 157-171.

32. Veenstra R, Lindenberg S, Oldehinkel AJ, De Winter AF, Ormel J (2006) Temperament, environment, and antisocial behavior in a population sample of preadolescent boys and girls Int J Behav Dev 30: 422-432.

33. Ozsoy S, Besirli A, Abdulrezzak U, Basturk M (2014) Serum ghrelin and leptin levels in patients with depression and the effects of treatment. Psychiatry Investig 11: 167-172.

34. Bender R, Lange S (2001) Adjusting for multiple testing - when and how? J Clin Epidemiol 54: 343-349.

35. Lanfumey L, Mongeau R, Cohen-Salmon C, Hamon M (2008) Corticosteroid–serotonin interactions in the neurobiological mechanisms of stress-related disorders. Neuroscience & Biobehavioral Reviews 32: 1174-1184.

36. Kormos V, Gaszner B (2013) Role of neuropeptides in anxiety, stress, and depression: From animals to humans. Neuropeptides 47: 401-419.

37. Spencer SJ, Xu L, Clarke MA, Lemus M, Reichenbach A, et al. (2012) Ghrelin regulates the hypothalamic-pituitary-adrenal axis and restricts anxiety after acute stress. Biol Psychiatry 72: 457-465.

38. Benso A, Calvi E, Gramaglia E, Olivetti I, Tomelini M, et al. (2013) Other than growth hormone neuroendocrine actions of ghrelin. Endocr Dev 25: 59-68.

39. Rossi F, Dietrich G, Kazatchkine MD (1989) Anti-idiotypes against autoantibodies in normal immunoglobulins: evidence for network regulation of human autoimmune responses. Immunol Rev 110: 135-149.

40. Deloumeau A, Bayard S, Coquerel Q, Déchelotte P, Bole-Feysot C, et al. (2010) Increased immune complexes of hypocretin autoantibodies in narcolepsy. PLoS One 5: e13320.

41. Schaefer JM, Fetissov SO, Legrand R, Claeyssens S, Hoekstra PJ, et al. (2013) Corticotropin (ACTH)-reactive immunoglobulins in adolescents in relation to antisocial behavior and stress-induced cortisol response. The TRAILS study. Psychoneuroendocrinology 38: 3039–3047.

42. Garcia FD, Coquerel Q, Kiive E, Déchelotte P, Harro J, et al. (2011) Autoantibodies reacting with vasopressin and oxytocin in relation to cortisol secretion in mild and moderate depression. Progress in Neuro-Psychopharmacology and Biological Psychiatry 35: 118-125.

43. Oldstone MB (1998) Molecular mimicry and immune-mediated diseases. Faseb J 12: 1255-1265.

44. Krumbholz A, Philipps A, Oehring H, Schwarzer K, Eitner A, et al. (2011) Current knowledge on PB1-F2 of influenza A viruses. Medical Microbiology and Immunology 200: 69-75.

45. Krejnusová I, Gocníková H, Bystrická M, Blaškovičová H, Poláková K, et al. (2009) Antibodies to PB1-F2 protein are induced in response to influenza A virus infection. Archives of Virology 154: 1599-1604.

46. Tennoune N, Chan P, Breton J, Legrand R, Chabane YN, et al. (2014) Bacterial ClpB heat-shock protein, an antigen-mimetic of the anorexigenic peptide [alpha]-MSH, at the origin of eating disorders. Transl Psychiatry 4: e458.

47. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, et al. (2001) A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7: 1306-1312.

48. Strachan E, Saracino M, Selke S, Magaret A, Buchwald D, et al. (2011) The effects of daily distress and personality on genital HSV shedding and lesions in a randomized, double-blind, placebo-controlled, crossover trial of acyclovir in HSV-2 seropositive women. Brain, Behavior, and Immunity 25: 1475-1481.

49. Luyt C-E, Combes A, Becquemin M-H, Beigelman-Aubry C, Hatem S, et al. (2012) Long-term outcomes of pandemic 2009 influenza a(h1n1)-associated severe ards. Chest 142: 583-592.

50. Okusaga O, Yolken RH, Langenberg P, Lapidus M, Arling TA, et al. (2011) Association of seropositivity for influenza and coronaviruses with history of mood disorders and suicide attempts. Journal of Affective Disorders 130: 220-225.

51. Iseme RA, McEvoy M, Kelly B, Agnew L, Attia J, et al. (2014) Autoantibodies and depression: Evidence for a causal link? Neuroscience & Biobehavioral Reviews 40: 62-79.

52. Kneeland RE, Fatemi SH (2013) Viral infection, inflammation and schizophrenia. Progress in Neuro-Psychopharmacology and Biological Psychiatry 42: 35-48.

# chapter 5

## Reciprocal associations between stressful life events and cannabis use during adolescence. The TRAILS study.

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## ABSTRACT

Stressful life events (SLE) may lead to increased cannabis use during adolescence, but they may also be a consequence of cannabis use. Considering the implications of this relation, we studied the reciprocal associations between SLE and cannabis use during adolescence and young adulthood in a prospective cohort of 2230 Dutch adolescents from the general population using cross-lagged path models. SLE were measured at age 16,19 and 2

Self-reported cannabis use in the past year was assessed at age 16, 19 and 2

SLE at age 16 predicted cannabis use at age 19 and vice versa, and cannabis use at age 19 predicted SLE at age 2

The strength of these relationships decreased after adjustment for externalizing behaviour. Internalizing behaviour, parental antisocial behaviour and parental substance use did not influence the associations. Our findings that SLE can predict cannabis use, and that cannabis use can predict SLE in adolescence and young adulthood could be of importance in identifying adolescents at risk and in guiding prevention efforts. The role of externalizing problems in this relationship needs to be further studied.

## INTRODUCTION

Drug use at a young age, especially regular and prolonged use, is an important risk factor for addiction and drug abuse later in life [1,2]. In particular, cannabis use during adolescence has been associated with later difficulties, e.g., reduced educational achievement, unemployment, and use of potentially more harmful drugs [3-7]. Stressful life events (SLE) may precede cannabis use, which in turn could lead to more SLE, creating a vicious cycle that is difficult to escape from. Considering that adolescence is a very vulnerable period to the negative effects of both cannabis use and SLE, and given the notion that the relationship between cannabis use and SLE might be bidirectional, it is important to investigate the reciprocal relationships between these two variables. In addition, identification of factors that contribute to the initiation and continuation of cannabis use during adolescence is important to guide prevention efforts.



SLE have been considered to play an important role in drug use initiation and the development of addiction. Some models suggest that drug use reduces the negative affect associated with stress and meanwhile enhances mood [8-10]. Indeed, studies in adults suggest a positive association between stress and drug use [9,11,12]. In adolescents, cross-sectional studies have shown that family stressors and relational victimization were associated with drug use, including cannabis [13-16]. However, most studies in adults assess childhood adversity retrospectively. The issue of biased recall of SLE is problematic and often leads to over- or underestimation of events, depending on the present state of mind when recalling the past [17]. Moreover, drug users can show memory disturbances [18]. To our knowledge, only two longitudinal studies so far have addressed the relationship between SLE and cannabis use. They found that drug use in early adulthood was more likely after maltreatment before the age of eleven [19], and that parental divorce and childhood neglect were not predictive of cannabis use in young adulthood [20]. While these two studies looked at childhood SLE, we are not aware of any longitudinal studies assessing SLE experienced in adolescence in relation to cannabis use.

The association between SLE and adolescent cannabis use is arguably complex. While SLE often precede drug use [19,20], drug use may also lead to more SLE during adolescence, e.g., problems at school and with peers, family and relationship conflicts, financial difficulties and health problems. This could in turn lead to the continuation or escalation of drug use. So far, few studies have investigated whether the relationship between SLE and drug use is reciprocal. One study that examined abuse and drug use in general in adult women showed that the relationship was bi-directional [21]. A prospective study in frequent cannabis users showed that recent SLE predicted the onset of cannabis dependence in young adults [22].

A challenge in this research area is that besides the complex association between SLE and drug use also other factors are associated with both phenomena. It is known that SLE and drug use share common risk factors, for example externalizing problems

[23-25], parental antisocial behavior and parental substance use [26-29]. Studies in the field of drug use often fail to adjust for common risk factors [6].

To summarize, studies support the notion of a positive relationship between SLE and drug use; however, they have not studied the reciprocal relationships between the two in a longitudinal manner. In this study we tested whether SLE were reciprocally related to cannabis use during adolescence and young adulthood. We hypothesized that more SLE would be associated with a higher likelihood of cannabis use, and that cannabis use would be associated with the subsequent occurrence of more SLE. Furthermore, we tested whether these associations were independent of common risk factors like externalizing and internalizing problems, parental substance use and parental antisocial behavior. For this study we used data from the TRacking Adolescents' Individual Lives Survey (TRAILS), a prospective cohort study of Dutch adolescents.



## METHODS

### Sample

In TRAILS, 2230 Dutch adolescents from the general population have been followed up biennially or tri-annually from age 10 onwards [30]. Participants were recruited from both urban and rural areas in the North of the Netherlands. To date, five assessment waves have taken place (T1 – T5). At T1, 2230 children (mean age=11.1 years, SD=0.55, 51% girls) enrolled in the study (response rate 76.0%, [31]). Of these 2230 participants, 96.4% (N=2149, mean age=13.6 years; SD=0.53) participated at T2, 81.4% (N=1816, mean age=16.3 years, SD=0.73,) participated at T3, 84.3% (N=1881, mean age=19.1 years; SD=0.60) participated at T4, and 68.4% (N=1525, mean age = 22.3 years; SD=0.65) participated at T5 [32]. At T3, 1513 adolescents participated in the Event History Calendar (EHC) interview, which was used to measure SLE between T1 and T1. The Events Checklist questionnaire was filled out by 1714 adolescents at T4 and by 1477 adolescents at T5 (see below).

### Measures

#### Cannabis use

Cannabis use was assessed with self-report questionnaires, filled out at school or at the participants' home. Confidentiality of the study was emphasized so that adolescents were reassured that their parents or teachers would not have access to the information they provided. Cannabis use at T3, T4 and T5 was assessed with the question: "How many times have you used weed (marijuana) or hash in the past year?". Answers were recoded into 'no use' versus 'at least once' during the past year.

### Stressful life events

SLE were assessed at T3 using the Event History Calendar (EHC), a reliable method for obtaining data about life events [33]. The calendar was adapted into a 45-minute

interview. Participants were asked about events that occurred since T1. At T4 and T5, adolescents filled out an Events Checklist questionnaire, which assessed stressful and non-stressful life events in the past two years. For each time point, we only included negative SLE, based on other studies about the impact of SLE on adolescents [15,34,35]. These SLE (T3: N=20, T4: N=22, T5: N=23). For example: death of a family member or partner, crime and involvement with police, sexual or physical abuse, rejection by friends or partners, financial problems and problems at school or work. SLE at T4 and T5 were the same, except for one event (bullying was not included in the T4 questionnaire). SLE at T3 overlapped partly with SLE at T4 and T5 (details of all included life events are available upon request). SLE were coded as present or not present and the Z-score of the sum of all SLE was used.



### Other variables

Age and sex were assessed at T1 Socioeconomic status (SES) was calculated as the mean of income, educational, and occupational level of each parent at T1 [36], and a Z-score was calculated [37]. Externalizing and internalizing problems were assessed with the Youth Self Report (YSR) at T2 and T3, and with the Adolescent Self Report (ASR) at T4, both filled out by the adolescent [38,39].

At T1, *lifetime parental antisocial behavior* was assessed during the 'Brief TRAILS Family History Interview'. If either mother or father had ever engaged in violence, theft, vandalism, committed a crime or used a weapon the variable 'parental antisocial behavior' was coded as yes, otherwise as no. *Daily parental smoking* was assessed at T1 and T3 and defined as no parent smoked, one parent smoked or both parents smoked daily during the past year (T1) or the past four weeks (T3). *Parental alcohol use* at T1 and T3 was assessed for each parent as none, 1-3 glasses, 4-10 glasses, 11-20 glasses and more than 20 glasses per week. If either mother or father had ever been addicted to an illicit drug, the variable *addiction* was coded as (1) yes, otherwise as (0) no.

### Statistical Analysis

Descriptives of the study sample were calculated in IBM SPSS Statistics (version 21). We used cross-lagged path modeling to investigate the reciprocal associations between SLE and cannabis use during adolescence and young adulthood (Figure 1). Analyses were done in Mplus version 7.31 [40]. Mplus uses full information maximum likelihood (FIML) to deal with missing values. To determine model fit, we used the comparative fit index (CFI) with values > .90 indicating acceptable fit and values > .95 indicating close fit [41], and the root mean square error of approximation (RMSEA), with values between .08 and .06 indicating acceptable fit and values < .06 indicating close fit [42]. First, cross-lagged path modeling was done adjusting only for the baseline covariates sex, age and socioeconomic status. This analysis included 1938 adolescents out of the 2230 who participated in our study (292 individuals had missing data on all variables). In a second step, we added other potential covariates: externalizing

behavior scores, internalizing behavior scores, parental smoking, parental alcohol use, parental addiction and parental problem behavior. In case the covariate added changed the regression coefficients by more than 10%, we added it to the model [43].

## RESULTS

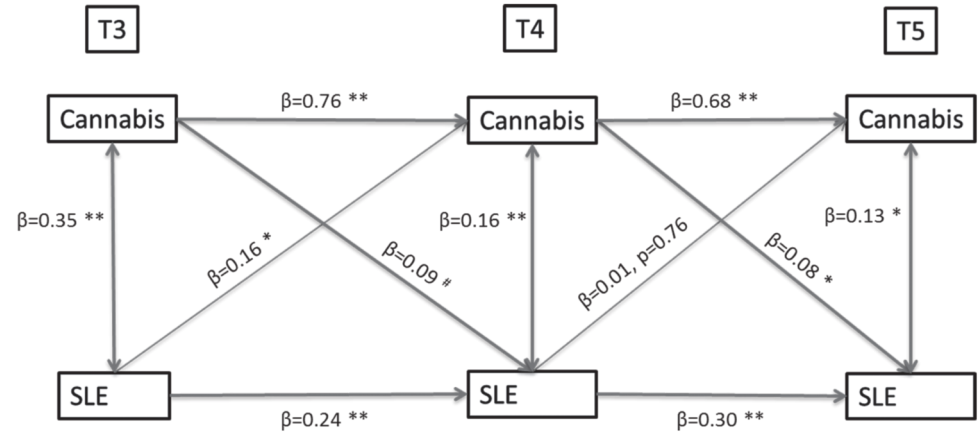


Descriptives of the study sample are presented in Table 1. Two models of the reciprocal associations between SLE and cannabis use are presented in Figure 1.The baseline model with the covariates sex, age, and socioeconomic status revealed reciprocal associations between cannabis use at age 16 and SLE at age 19 and vice versa (Figure 1A). We also found a positive association between cannabis use at age 19 and SLE at age 22, but not between SLE at age 19 and cannabis use at age 22 (Figure 1A). The model including baseline covariates showed a good fit, with a CFI of .99 and a RSMEA of .0 Internalizing problems, parental smoking, parental alcohol use and parental problem behavior had no effect on the reciprocal associations between SLE and cannabis use and were therefore not included in our final model. When adding adolescents' externalizing problems, the cross-lagged path coefficients changed and became non-significant (Figure 1B). The model fit was acceptable, with a CFI of .91 and a RSMEA of .0 The final model included the baseline covariates plus externalizing behavior as covariates and data of 1910 adolescents was included in this analysis (28 individuals had missing data on all covariates).

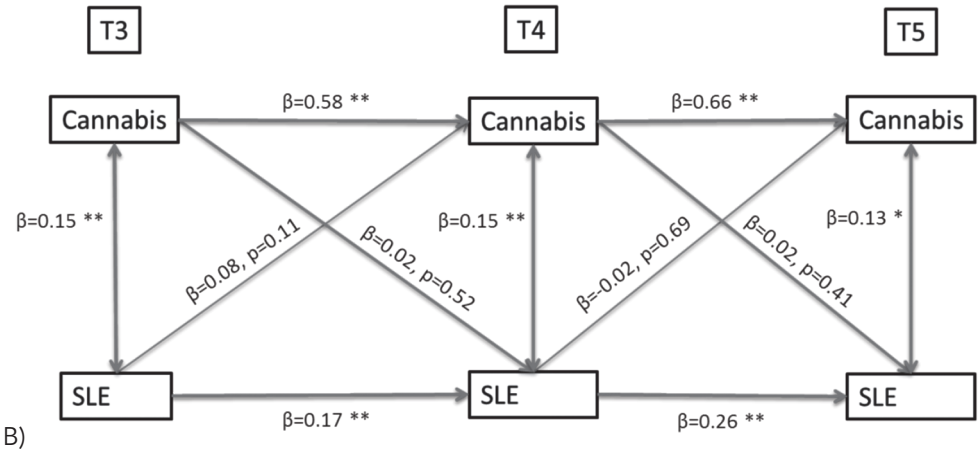
We also found significant cross-sectional correlations between SLE and cannabis use at each time point, as well as associations between cannabis use at T3 and cannabis use at T4 and subsequently at T5.Similarly, the mean number of SLE at different time points were strongly related. These associations persisted after adjustment for the covariates we measured in this study (Figure 1B).

**Table 1:** Study participants

| | N (%) | Mean (SD) |
|---|---|---|
| Total sample | 2230 | |
| Sex (% boys) | 1098 (49.2%) | |
| Cannabis use in the past year | | |
| Cannabis use at T3 | 397 (17.8%) | |
| Cannabis use at T4 | 586 (26.3%) | |
| Cannabis use at T5 | 519 (23.3%) | |
| Mean number of stressful life events | | |
| T3 | | 4.7 (2.6) |
| T4 | | 2.2 (1.9) |
| T5 | | 3.0 (2.2) |
| Age in years | | |
| T3 | | 16.3 (0.7) |
| T4 | | 19.1 (0.6) |
| T5 | | 22.3 (0.7) |




A)




B)

**Figure 1: Reciprocal associations between stressful life events (SLE) and cannabis use.** Cross-lagged path model showing associations between SLE (z-scores) and cannabis use in the past year at three time points (T3= mean age 16, T4= mean age 19, T5= mean age 22), adjusted for sex, age and socioeconomic status (A, N=1938) and adjusted for sex, age, socioeconomic status and externalizing behavior (B, N=1910). # p<0.05; * p<0.01; ** p<0.001

## DISCUSSION

We examined the reciprocal relationship between SLE and cannabis use in a large cohort study of 2230 adolescents (TRAILS). We found that SLE were associated with subsequent cannabis use during adolescence and young adulthood and vice versa. These associations were attenuated by adding externalizing problem scores to our model.

The result that SLE predict cannabis use in adolescence is in agreement with previous cross-sectional studies that showed a positive association between SLE and drug use [11,12]. One way to interpret this association is to consider drug use as

a possible stress-coping mechanism that aims to reduce negative affect and to increase positive affect [9,10,44]. Our study confirms this relationship, and thereby suggests that cannabis use in adolescents who experienced multiple SLE could serve as a coping mechanism to alleviate stress, emotional or physical pain induced by the SLE [9]. However, SLE may also be related to drug use through other mechanisms. Family stress, for example, could be related to ineffective parenting (e.g., authoritarian parenting, rejection, lack of monitoring), which presents a higher risk for substance use in adolescence [45,46]. Family stress may also stimulate peer orientation [47] which in turn might lead to the involvement with delinquent peers and increased susceptibility to antisocial peer pressure and risky decision-making. In contrast to early adolescence, we did not find any association between SLE at age 19 and cannabis use at age 2.



An explanation could be that while SLE play an important role in the initiation of cannabis use or in 'early stages', the continuation of cannabis use in young adulthood, and possibly the development of addiction, may be influenced by other factors, arguably in combination with SLE. These factors might include neurobiological mechanisms, genetic susceptibility, stress biology, coping mechanisms and enhanced craving [9,48]. Cannabis use was also associated with the subsequent occurrence of SLE, both in adolescence and young adulthood, suggesting that the relationship between SLE and cannabis use is bi-directional. This is plausible, as cannabis use could lead to involvement with police, criminal behavior, financial problems, conflicts at school, with peers and with family members.

After adjustment for externalizing problems, the reciprocal associations between cannabis use and SLE were attenuated, while internalizing problems did not change the associations. It is difficult to discern different risk behaviors in adolescence, e.g., externalizing behavior and cannabis use, as they are highly correlated. Thus, one possible explanation is that the relationships we found are not specific for cannabis use but represent associations between SLE and a more general ´externalizing behavior profile´. In line with this, higher levels of externalizing problems, but not internalizing problems, have been reported in frequent cannabis users than in the general population [49]. Another study has shown that externalizing behavior mediated the relationship between SLE and drug addiction in young adulthood [50]. In support of this notion, a previous TRAILS study reported that externalizing behavior precedes cannabis use in young adolescents, while internalizing behavior did not have any influence on cannabis use [51]. We therefore have to be careful in interpreting this finding, as externalizing behavior might lie in the path between SLE and cannabis use. Parental substance use and antisocial behavior did not affect the reciprocal associations between SLE and cannabis use in our study. However, they have been shown to be risk factors for adolescent substance use [26]. It is possible that they play a role as moderators in the relationship between SLE and cannabis use.

Our study has several strengths. Most importantly, it is one of the first studies to investigate the reciprocal associations between SLE during adolescence and cannabis

use in the same population, using longitudinal and prospective data from multiple measurement waves. SLE were assessed over the past years, but at three different time points (age 16, 19 and 22), which allows a prospective study of the relationship with cannabis use and probably provides more reliable results than if childhood SLE are assessed later in adulthood, which is often the case in retrospective studies. We did not focus only on one SLE but assessed a comprehensive list of SLE which were adapted to the age group, to capture the combined effect of negative SLE on cannabis use. In addition, our study measured and tested the influence of several common risk factors for both SLE and cannabis use.



The present study has some limitations. Due to our study's cohort design, selective attrition is to be expected. While we used the method of full information maximum likelihood to deal with missing data, some variables had missing values on all variables and were therefore not included in the analyses. Therefore, we cannot guarantee that possible bias due to missing data was fully resolved. Our study relied on self-reported data, which carries the risk of being falsely reported, although studies have found self-reported data on alcohol and drug use to be relatively reliable [52,53]. While about half of the SLE measured by the Event History Calendar at T3 and the events checklist questionnaire at T4 and T5 were the same, discrepancies could have led to different associations with cannabis use. The cross-sectional correlations between SLE and cannabis use were stronger than the cross-lagged paths in our model, maybe due to an immediate increase in cannabis use after the occurrence of an SLE. In turn, it is also plausible that cannabis use could directly be followed by an SLE, for example contact with police or a fight with parents immediately after cannabis use. These ´immediate´ short-term prospective associations could not be captured in our study. In addition, the transition from no cannabis use to regular cannabis use is probably determined by other factors than the transition from regular cannabis use to problematic cannabis use [54]. Here, we did not differentiate between irregular, regular, non-problematic and problematic cannabis use. It would be interesting to know whether SLE play a role particularly in whether adolescents develop a problematic pattern of cannabis use.

The present study shows that SLE and cannabis use during adolescence are reciprocally related. The role of externalizing problems in this relationship needs to be further studied. Future studies should explore factors that predict the transition from regular to problematic cannabis use during adolescence as adolescents are a vulnerable group for initiation as well as escalation of drug use. Our study suggests that measuring SLE can provide valuable information to design intervention programs and identify adolescents at risk of cannabis use. In addition, the finding that cannabis users tend to experience more SLE than non-users is important, as SLE are both a burden on adolescents and an important risk factor for mental health problems [55,56] and school problems [57].

## References


1. Fergusson DM, Horwood LJ, Swain-Campbell N (2002) Cannabis use and psychosocial adjustment in adolescence and young adulthood. Addiction 97: 1123-1135.
2. Behrendt S, Wittchen HU, Hofler M, Lieb R, Beesdo K (2009) Transitions from first substance use to substance use disorders in adolescence: Is early onset associated with a rapid escalation? Drug and Alcohol Dependence 99: 68-78.
3. Kandel DB, Yamaguchi K, Klein LC (2006) Testing the gateway hypothesis. Addiction 101: 470-472.
4. Agrawal A, Grant JD, Waldron M, Duncan AE, Scherrer JF, et al. (2006) Risk for initiation of substance use as a function of age of onset of cigarette, alcohol and cannabis use: findings in a Midwestern female twin cohort. Prev Med 43: 125-128.
5. Lynskey MT, Heath AC, Bucholz KK, Slutske WS, Madden PAF, et al. (2003) Escalation of drug use in early-onset cannabis users vs co-twin controls. Jama-Journal of the American Medical Association 289: 427-433.
6. Macleod J, Oakes R, Copello A, Crome I, Egger M, et al. (2004) Psychological and social sequelae of cannabis and other illicit drug use by young people: a systematic review of longitudinal, general population studies. Lancet 363: 1579-1588.
7. Arria AM, Garnier-Dykstra LM, Cook ET, Caldeira KM, Vincent KB, et al. (2013) Drug use patterns in young adulthood and post-college employment. Drug and Alcohol Dependence 127: 23-30.
8. Koob GF, LeMoal M (1997) Drug abuse: Hedonic homeostatic dysregulation. Science 278: 52-58.
9. Sinha R (2001) How does stress increase risk of drug abuse and relapse? Psychopharmacology 158: 343-359.
10. Khantzian EJ (1985) The self-medication hypothesis of addictive disorders: focus on heroin and cocaine dependence. Am J Psychiatry 142: 1259-1264.
11. Douglas KR, Chan G, Gelernter J, Arias AJ, Anton RF, et al. (2010) Adverse childhood events as risk factors for substance dependence: Partial mediation by mood and anxiety disorders. Addictive Behaviors 35: 7-13.
12. Ompad DC, Ikeda RM, Shah N, Fuller CM, Bailey S, et al. (2005) Childhood sexual abuse and age at initiation of injection drug use. Am J Public Health 95: 703-709.
13. Butters JE (2002) Family stressors and adolescent cannabis use: a pathway to problem use. Journal of Adolescence 25: 645-654.
14. Sullivan TN, Farrell AD, Kliewer W (2006) Peer victimization in early adolescence: association between physical and relational victimization and drug use, aggression, and delinquent behaviors among urban middle school students. Dev Psychopathol 18: 119-137.
15. Windle M, Wiesner M (2004) Trajectories of marijuana use from adolescence to young adulthood: Predictors and outcomes. Development and Psychopathology 16: 1007-1027.
16. Low NCP, Dugas E, O'Loughlin E, Rodriguez D, Contreras G, et al. (2012) Common stressful life events and difficulties are associated with mental health symptoms and substance use in young adolescents. Bmc Psychiatry 12.
17. Dellafemina D, Yeager CA, Lewis DO (1990) Child-Abuse - Adolescent Records Vs Adult Recall. Child Abuse & Neglect 14: 227-231.
18. Solowij N, Jones KA, Rozman ME, Davis SM, Ciarrochi J, et al. (2011) Verbal learning and memory in adolescent cannabis users, alcohol users and non-users. Psychopharmacology (Berl) 216: 131-144.

19. Huang S, Trapido E, Fleming L, Arheart K, Crandall L, et al. (2011) The long-term effects of childhood maltreatment experiences on subsequent illicit drug use and drug-related problems in young adulthood. Addictive Behaviors 36: 95-102.

20. Silins E, Hutchinson D, Swift W, Slade T, Toson B, et al. (2013) Factors associated with variability and stability of cannabis use in young adulthood. Drug and Alcohol Dependence 133: 452-458.

21. Kilpatrick DG, Acierno R, Resnick HS, Saunders BE, Best CL (1997) A 2-year longitudinal analysis of the relationships between violent assault and substance use in women. Journal of Consulting and Clinical Psychology 65: 834-847.

22. van der Pol P, Liebregts N, de Graaf R, Korf DJ, van den Brink W, et al. (2013) Predicting the transition from frequent cannabis use to cannabis dependence: a three-year prospective study. Drug Alcohol Depend 133: 352-359.

23. Sitnick SL, Shaw DS, Hyde LW (2014) Precursors of adolescent substance use from early childhood and early adolescence: testing a developmental cascade model. Dev Psychopathol 26: 125-140.

24. Miettunen J, Murray GK, Jones PB, Maki P, Ebeling H, et al. (2013) Longitudinal associations between childhood and adulthood externalizing and internalizing psychopathology and adolescent substance use. Psychol Med: 1-12.

25. Monshouwer K, Harakeh Z, Lugtig P, Huizink A, Creemers HE, et al. (2012) Predicting transitions in low and high levels of risk behavior from early to middle adolescence: the TRAILS study. J Abnorm Child Psychol 40: 923-931.

26. Li C, Pentz MA, Chou CP (2002) Parental substance use as a modifier of adolescent substance use risk. Addiction 97: 1537-1550.

27. Roettger ME, Swisher RR, Kuhl DC, Chavez J (2011) Paternal incarceration and trajectories of marijuana and other illegal drug use from adolescence into young adulthood: evidence from longitudinal panels of males and females in the United States. Addiction 106: 121-132.

28. Hoffmann JP, Cerbone FG (2002) Parental substance use disorder and the risk of adolescent drug abuse: an event history analysis. Drug and Alcohol Dependence 66: 255-264.

29. Hoffmann JP, Su SS (1998) Parental substance use disorder, mediating variables and adolescent drug use: a non-recursive model. Addiction 93: 1351-1364.

30. Ormel J, Oldehinkel AJ, Sijtsema J, van Oort F, Raven D, et al. (2012) The TRacking Adolescents' Individual Lives Survey (TRAILS): design, current status, and selected findings. J Am Acad Child Adolesc Psychiatry 51: 1020-1036.

31. de Winter AF, Oldehinkel AJ, Veenstra R, Brunnekreef JA, Verhulst FC, et al. (2005) Evaluation of non-response bias in mental health determinants and outcomes in a large sample of pre-adolescents. European Journal of Epidemiology 20: 173-181.

32. Nederhof E, Jorg F, Raven D, Veenstra R, Verhulst FC, et al. (2012) Benefits of extensive recruitment effort persist during follow-ups and are consistent across age group and survey method. The TRAILS study. BMC Med Res Methodol 12: 93.

33. Caspi A, Moffitt TE, Thornton A, Freedman D, Amell JW, et al. (1996) The life history calendar: A research and clinical assessment method for collecting retrospective event-history data. International Journal of Methods in Psychiatric Research 6: 101-114.

34. Laceulle OM, Nederhof E, Karreman A, Ormel J, Van Aken MAG (2012) Stressful Events and Temperament Change during Early and Middle Adolescence: The TRAILS Study. European Journal of Personality 26: 276-284.

35. McMahon SD, Grant KE, Compas BE, Thurm AE, Ey S (2003) Stress and psychopathology in children and adolescents: is there evidence of specificity? J Child Psychol Psychiatry 44: 107-133.

36. Ganzeboom HBG, Treiman DJ (1996) Internationally comparable measures of occupational status for the 1988 International Standard Classification of Occupations. Social Science Research 25: 201-239.

37. Veenstra R, Lindenberg S, Oldehinkel AJ, De Winter AF, Ormel J (2006) Temperament, environment, and antisocial behavior in a population sample of preadolescent boys and girls. International Journal of Behavioral Development 30: 422-432.

38. Achenbach TM (1991) Manual for the youth self-report and 1991 profile. . Burlington: University of Vermont Department of Psychiatry.

39. Verhulst FC, van der Ende, J., Koot, H.M. (1997) Handleiding voor de Youth Self-Report (YSR). Afdeling Kinder- en Jeugdpsychiatrie Sophia Kinderziekenhuis/ Academisch Ziekenhuis Rotterdam/ Erasmus Universiteit Rotterdam, Rotterdam.

40. Muthén BO, Muthén LK (1998-2012) Mplus User´s Guide. 7th edition Los Angeles, CA.

41. Bentler PM, Bonett DG (1980) Significance Tests and Goodness of Fit in the Analysis of Covariance Structures. Psychol Bull 88: 588-606.

42. Browne MW, Cudeck R (1993) Alternative ways of assessing model fit. In K A Bollen & J S Long (Eds) Testing structural equation models 136-162.

43. Nelson MC, Gordon-Larsen P, Adair LS (2005) Are adolescents who were breast-fed less likely to be overweight? Analyses of sibling pairs to reduce confounding. Epidemiology 16: 247-253.

44. Wong CF, Silva K, Kecojevic A, Schrager SM, Bloom JJ, et al. (2013) Coping and emotion regulation profiles as predictors of nonmedical prescription drug and illicit drug use among high-risk young adults. Drug and Alcohol Dependence 132: 165-171.

45. Neiderhiser JM, Marceau K, Reiss D (2013) Four factors for the initiation of substance use by young adulthood: a 10-year follow-up twin and sibling study of marital conflict, monitoring, siblings, and peers. Dev Psychopathol 25: 133-149.

46. Bohnert KM, Anthony JC, Breslau N (2012) Parental Monitoring at Age 11 and Subsequent Onset of Cannabis Use Up to Age 17: Results From a Prospective Study. Journal of Studies on Alcohol and Drugs 73: 173-177.

47. Fuligni AJ, Eccles JS (1993) Perceived Parent-Child Relationships and Early Adolescents Orientation toward Peers. Developmental Psychology 29: 622-632.

48. Hiroi N, Agatsuma S (2005) Genetic susceptibility to substance dependence. Mol Psychiatry 10: 336-344.

49. van der Pol P, Liebregts N, de Graaf R, Ten Have M, Korf DJ, et al. (2013) Mental health differences between frequent cannabis users with and without dependence and the general population. Addiction 108: 1459-1469.

50. King KM, Chassin L (2008) Adolescent stressors, psychopathology, and young adult substance dependence: a prospective study. J Stud Alcohol Drugs 69: 629-638.

51. Griffith-Lendering MF, Huijbregts SC, Mooijaart A, Vollebergh WA, Swaab H (2011) Cannabis use and development of externalizing and internalizing behaviour problems in early adolescence: A TRAILS study. Drug Alcohol Depend 116: 11-17.

52. Del Boca FK, Darkes J (2003) The validity of self-reports of alcohol consumption: state of the science and challenges for research. Addiction 98: 1-12.

53. Del Boca FK, Noll JA (2000) Truth or consequences: the validity of self-report data in health services research on addictions. Addiction 95: S347-S360.

54. von Sydow K, Lieb R, Pfister H, Hofler M, Wittchen HU (2002) What predicts incident use of cannabis and progression to abuse and dependence? A 4-year prospective examination of risk factors in a community sample of adolescents and young adults. Drug Alcohol Depend 68: 49-64.

55. Amone-P'Olak K, Ormel J, Huisman M, Verhulst FC, Oldehinkel AJ, et al. (2009) Life stressors as mediators of the relation between socioeconomic position and mental health problems in early adolescence: the TRAILS study. J Am Acad Child Adolesc Psychiatry 48: 1031-1038.

56. Bakker MP, Ormel J, Verhulst FC, Oldehinkel AJ (2011) Adolescent family adversity and mental health problems: the role of adaptive self-regulation capacities. The TRAILS study. J Abnorm Child Psychol 39: 341-350.

57. DuBois DL, Felner RD, Brand S, Adan AM, Evans EG (1992) A prospective study of life stress, social support, and adaptation in early adolescence. Child Dev 63: 542-557.

# chapter 6

## Do stress responses in adolescence predict problematic cannabis use in young adulthood? The TRAILS study.

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## Abstract

Blunted stress reactivity reflects a higher risk of initiating cannabis use in adolescence. However, it is unknown whether stress reactivity is associated with problematic cannabis use. We studied whether the physiological stress response and perceived arousal to a social stress paradigm in adolescence were predictive of the development of problematic cannabis use in early adulthood. We further studied whether stress reactivity played a role in whether cannabis users develop problematic cannabis use.

We measured levels of cortisol and perceived arousal, both at baseline and in response to a social stress paradigm in 715 adolescents (mean age 16) of the general population. Problematic cannabis use was assessed with the Cannabis Use Problems Identification Test (CUPIT) at a mean age of 22. Analyses were adjusted for general risk factors of problematic cannabis use, such as internalizing and externalizing behaviour and the use of other substances. The same analyses were done selecting only adolescents who already used cannabis at age 16.

Basal cortisol levels and cortisol responses to stress were not associated with later problematic cannabis use in the total sample. However, in adolescents who already used cannabis at age 16, high perceived arousal in anticipation of a stressor was associated with a higher risk of problematic cannabis use at follow-up (OR=2.36; CI=1.01; 5.52, $p<0.05$).

Our results suggest that stress reactivity does not predict development of problematic cannabis use in adolescents of the general population. Future research should focus on the important question of predicting which cannabis users develop problematic cannabis use.

## Introduction

Cannabis is the most commonly used illicit drug worldwide and it is frequently used in adolescence. Frequent cannabis use can lead to *problematic* cannabis use and the development of a cannabis use disorder (CUD), which is characterized by dependence, withdrawal symptoms, difficulties at school or work and with family and friends, financial problems and health problems [1]. Around 9% of those who use cannabis regularly develop a CUD [2]. In the Netherlands, 13.3% of 16 year-olds reported cannabis use in the past month and 22.8% in the past year in 2013 [3], which is slightly higher than in other European countries [4]. In the general population, 1.4%, met criteria for a CUD according to DSM IV [5]. Adolescents are very vulnerable to the effects of cannabis because the brain is still developing and regular cannabis use during this period has been associated with neurocognitive problems and increased risk for later drug problems [6-9]. Multiple risk factors for cannabis use are known, for example, sensation seeking, externalizing behaviors, stressful life events and use of other substances [10-13]. However, the biological mechanisms underlying *problematic* cannabis use in particular are not fully understood. Another important questions that still needs to be answered is "Why do some cannabis users develop a problematic pattern of cannabis use while others don't?". Thus, it is of high importance to investigate the physiological and psychological mechanisms behind the development of problematic cannabis use.



A high tendency for novelty or sensation seeking has been associated with higher prevalence of cannabis use [14,15]. The underlying explanation could be that adolescents with a high tendency for novelty or sensation seeking experience a constant state of physiological under-arousal, and engage in substance use to achieve a pleasant state of arousal [16,17]. The hypothalamic-pituitary-adrenal (HPA) axis is one of the physiological stress systems that has been implicated in substance use. The acute and chronic use of cannabis or other substances induces activation of the HPA axis [18-20]. On the other hand, down-regulation of the HPA axis resulting in under-arousal may be a precursor of regular and maybe problematic substance use. A previous study that used data from the Tracking Adolescents' Individual Lives Survey (TRAILS), a longitudinal general population study, found that a blunted cortisol awakening response (CAR) at the age of 11 predicted early -before the age of 13- initiation of cannabis use [21]. In addition, another TRAILS study showed that a blunted HPA axis reactivity in response to a social stress paradigm was associated with current cannabis use in 16-year olds [22]. Further, Moss et al. found that hypo-reactivity of the HPA axis in anticipation of a stressor was associated with regular monthly cannabis use in a sample of high-risk boys [16]. These findings suggest that blunted activity of the HPA-axis is associated with cannabis use; however, the association with *problematic* cannabis use has not been studied thus far. Similarly, blunted perceived arousal could also be related to an increased urge to seek out external stimulation, for example by using cannabis. To our knowledge there are no studies so far that have investigated the relationship between perceived arousal and cannabis use.

Therefore, our aim was to prospectively study the association between both physiological stress and perceived arousal at baseline and in response to a social stress paradigm at mean age 16 and problematic cannabis use at follow-up at mean age 22. In addition, we tested whether physiological stress or perceived arousal could predict the development of problematic cannabis use at age 22 in adolescents who already used cannabis at age 16. Based on previous studies [21,22] addressing the initiation of cannabis use, we hypothesized that blunted baseline levels and a blunted cortisol response to a social stress paradigm, as well as blunted perceived arousal, would increase adolescents' risk of developing *problematic* cannabis use.



## Study

### Sample

The current study is part of TRAILS, a prospective cohort study of Dutch adolescents, who have been followed up biennially or tri-annually from age 10 onwards [23]. The present study mainly involves data from the third (T3) and fifth (T5) assessment. Data from the other assessment waves were used solely for carrying out multiple imputation to predict missing values (see statistical analyses). At T1, 2230 children (mean age = 11.1 years, SD = 0.55, 51% girls) enrolled in the study (response rate 76.0%, [24]). Of these 2230 participants, 96.4% (N = 2149, mean age 13.6 years; SD 0.53) participated at T2, 81.4% (N = 1816, mean age = 16.3 years, SD 0.73,) participated at T3, 84.3% (N = 1881, mean age = 19.1 years; SD = 0.60) participated at T4 [25] and 68.4% (N=1525, mean age = 22.3 years; SD=0.65) participated at T5. At T3, 744 adolescents were invited to perform a series of laboratory tasks (experimental session) on top of the usual assessments, and 715 (96.1%) agreed to do so. The costly and labor-intensive nature of the laboratory tasks precluded assessing the whole sample. Adolescents with an elevated risk of mental health problems had a greater chance of being selected for the experimental session. Elevated risk was defined based on T1 measures of temperament (high frustration and fearfulness, low effortful control), lifetime parental psychopathology, and living in a single-parent family. In total 66.0% of the focus sample had at least one of the above-described risk factors; the remaining 34.0% were selected randomly from the low-risk TRAILS participants. The focus sample still represented the whole range of problems seen in a normal population of adolescents [26]. In total, 715 (351 boys) took part in the experimental session. Adolescents using corticosteroids (n=7) were excluded from the cortisol analyses. In 600 adolescents, basal morning cortisol levels were measured on the day of the experimental session. Out of the 715 adolescents who participated in the GSST at T3, 606 participated in the study at T5. Adolescents who did not take part in the T5 measurements differed from those who did: they were more likely boys (60.6% vs. 47.9%, $p<0.01$), had a lower socio-economic status (mean=-0.22 vs. 0.12, $p<0.01$) were more likely to be smokers at T3 (34.9% vs. 22.1%, $p<0.01$) and had higher scores for perceived arousal in anticipation of the social stress test (mean=3.0

vs. 2.6, $p<0.05$). The cortisol measures did not differ between T5 participants and T5 non-participants.

### The social stress test

During the experimental session, participants' physiological responses (cortisol) and psychological responses (perceived arousal) to a variety of challenging conditions were recorded, including a modified version of the Trier Social Stress Test: the Groningen Social Stress Test (GSST)[23,27]. The experimental protocol was approved by the Central Committee on Research Involving Human subjects.



The experimental session took place in soundproof rooms with blinded windows, started between 08:00 and 09:30 or between 12:00 and 14:30 and lasted about three hours and 15 minutes. At the start of the session a short checklist was used to assess current medication use, including oral contraceptives (OC). Participants were asked to refrain from smoking and from having coffee, milk, chocolate, and other sugar-containing foods in the two hours before the session. Although free salivary cortisol levels are higher in the morning due to the circadian rhythm of cortisol production, morning and afternoon cortisol responses to social stress were expected [28] and found to be comparable in this sample [27]. For the GSST, participants were instructed to prepare a six-minute speech about themselves and deliver this speech in front of a video camera. They were told that their videotaped performance would be judged on content of speech as well as on use of voice and posture, and rank-ordered by peers after the experiment. The test assistant watched the performance critically, without showing empathy or encouragement. After the speech, a three minute rest interlude followed. Then, participants were instructed to subtract 17 repeatedly, starting with 13,278, while the test assistant gave negative feedback. This mental arithmetic task lasted six minutes, followed by three minutes of silence, after which the participants were thoroughly debriefed about the experiment.

## Measures

### Cannabis use

Cannabis use was assessed at each measurement wave (T1-T5) with self-report questionnaires, which were filled out at school or at the participants' home. Confidentiality of the study was emphasized so that adolescents were reassured that their parents or teachers would not have access to the information they provided. Cannabis use at T1 and T2 was assessed as lifetime use. Cannabis use at T3, T4 and T5 was assessed with the question: "How many times have you used weed (marijuana) or hash in the past year?" The possible answers were: no use, once, twice and so on, up to 10 times, 11 to 19 times, 20-39 times and 40 times and more. Cannabis use at T3 and T5 was defined as having used cannabis at least once in the past year.

Problematic cannabis use, indicating a current CUD or high risk for progression to a CUD, was assessed with the Cannabis Use Problems Identification Test (CUPIT)

[29]. The CUPIT is a self-report rating scale to screen for problematic cannabis use among both adolescents and adults. High scores on the CUPIT predict development of a CUD over a 12-months period and can identify problematic cannabis use in adolescents at risk as well as in the general population [29]. All adolescents who mentioned in the self-report questionnaire at T5 that they had used cannabis in the past year were asked to fill in the CUPIT questionnaire. The CUPIT consists of 16 items that refer to cannabis use and related problems over the past 12 months (e.g., "How often have you used cannabis first thing in the morning?", "Have you found it difficult to get through a day without using cannabis?"). The sum score was used in analyses. For analyses regarding problematic cannabis use, we split up adolescents who filled in the CUPIT questionnaire into two groups: 1) non-problematic users with scores up to 19 and 2) problematic users with scores equal to 20 or higher, according to Bashford et al. [29].



## Cortisol

Baseline HPA axis activity was assessed by taking one saliva sample in the morning. The cortisol awakening response (CAR) was calculated by subtracting the first from the second cortisol sample concentration, which was taken 30 min later. Adolescents were instructed not to eat, brush their teeth, or engage in heavy exercise during this half hour, and to bring the tubes with them to the test location. Adolescents who had taken medication that affects cortisol levels (corticosteroids, selective serotonin reuptake inhibitors) were excluded (n= 7).

Reactivity of the HPA axis during the GSST was assessed by four saliva cortisol samples, considering the normal delay (20-25 minutes) in peak cortisol response to experimental stressors [30]: (1) before the test, representing pre-test 'anticipation' cortisol levels, (2) directly after the test, reflecting HPA axis reactivity during speech, (3) 20 minutes after the test, reflecting levels at the end of the test, and (4) 40 minutes after the end of the GSST, representing post-test cortisol levels. The amount of cortisol released during the GSST ('reactivity' = incremental area under the curve with respect to increase, AUCincr) was calculated over these four measures, using the trapezoid formula described by Janssens et al. [31]. 'Recovery' was calculated by subtracting post-test values from the highest value during the test. Z-scores of the cortisol measures were used in the analyses.

Samples were stored at -20°C until analysis. Cortisol was assessed from saliva by the Salivette sampling device (Sarstedt, Numbrecht, Germany). Cortisol concentrations were determined in duplicates from 100µl saliva, using an in-house radioimmunoassay, applying a polyclonal rabbit cortisol antibody and 1,2,6,7 $^{3}$H cortisol (Amersham, Arlington Heights, IL). After incubation at 60°C, the bound and free fractions were separated using active charcoal. The intra-assay coefficient of variation was 8.2% for concentrations of 1.5nM, 4.1% for concentrations of 15nM, and 5.4% for concentrations of 30nM. The inter-assay coefficients of variation were, respectively, 12.6%, 5.6%, and 6.0%. The detection limit was 0.9nM.

## Perceived arousal

Baseline levels of perceived arousal were assessed with the short Profile of Mood Scale [32]. We used the tension scale (including six items describing current mood: nervous, panic, tense, restless, anxious, insecure; Cronbach's alpha = 0.69) as a measure for perceived arousal. It was rated at the beginning of the experimental session on a 5-point scale (1= not at all, 2= a little, 3= partly, 4= kind of, 5= very much).

Perceived arousal during the GSST was assessed by means of the Self-Assessment Manikin (SAM), a nonverbal pictorial assessment technique to measure the perceived arousal associated with a person's affective reaction to a stimulus [33]. The subjective intensity of arousal could be indicated by choosing one out of nine ordered pictures. The pictures were translated into a 9-point scale (range 1–9) in such a way that high scores represented high levels of arousal. Perceived arousal during the GSST was assessed directly after the test, with a reference to the test (How did you feel during this test?). Pre- and post-test perceived arousal was measured at the start (after 40 min of rest) and at the end of the experimental session (40 min after the GSST). SAM ratings for arousal have been shown to correlate almost perfectly (0.95) with corresponding scale of the Semantic Differential Scale [34]. We used the z-scores of the arousal measures in the analyses.



## Covariates

Sex and socioeconomic status were assessed at T1. Socioeconomic status was calculated as the z-score of the mean of income, educational, and occupational level of both parents at T1. Age, use of oral contraception and use of medication were assessed before the GSST at T3. Substance use (alcohol, cigarettes, cannabis) was assessed with a questionnaire at T3, on average around 3 months before the experimental session. Alcohol use was defined as having used alcohol on at least 4 occasions during the past four weeks (n=276). We distinguished between non-smokers and habitual smokers (>= 1 cigarette per week, n= 176). Cannabis use was defined as having used cannabis at least once in the past year (n=189). Internalizing and externalizing problems were measured with the Youth Self-Report at T3 [35].

## Data analysis

To reduce bias resulting from non-response at T5, we used multiple imputation of missing values [36]. To predict missing cannabis use data and CUPIT scores, we used data from T1 to T4 (i.e. cannabis use; smoking and alcohol use; externalizing and internalizing problems; socioeconomic status) as well as all other variables that we used in our analyses. If cannabis use at T5 was zero after the imputation, then the CUPIT score was also set to zero. Covariates were also imputed, they all had less than 5% missing values.

To analyze the association between physiological or psychological stress reactivity and future cannabis use, we first performed logistic regression analyses with HPA axis or perceived arousal measures at T3 as predictors and cannabis use (yes/ no) at T5 as outcome. Second, we used the CUPIT score of adolescents who had used cannabis in the past year at T5 as continuous outcome variable and performed linear regression analyses with the different stress reactivity and baseline measures at T3 as predictors. In addition, we tested with logistic regression whether there were any associations between HPA axis and perceived arousal measures and problematic cannabis use compared to non-problematic use.



Next we constructed models with covariates. In the minimally adjusted models we included sex and age as covariates. In the adjusted models we entered all covariates. Then we tested whether HPA axis or perceived arousal measures were associated with the development of problematic cannabis use in T3 cannabis users using a logistic regression model adjusting for all covariates. We tested interaction terms between stress reactivity measures and sex, and if significant, stratified analyses by sex.

## Results

At follow-up 258 young adults (36.1% out of the 715 respondents) had used cannabis in the past year. CUPIT scores were available for 232 young adults (mean score of 13.8; SD=14.0; see Table 1). The discrepancy in numbers resulted from multiple imputation of two different variables: Cannabis use and CUPIT score. Out of those with a CUPIT score, 54 adolescents had a score of 20 or higher and were defined as problematic users.

Boys showed higher HPA axis reactivity during the GSST than girls, and also reported higher perceived arousal. Adolescents who used cannabis at T3 were more likely to also use cannabis at T5, and as reported in a previous study, showed a blunted cortisol response during the GSST [22].

We did not find any significant associations between HPA axis reactivity measures at T3 and cannabis use (yes/no) at T5, nor with CUPIT scores or problematic cannabis use (Table 2). Sex did not moderate the relationship between physiological stress and later problematic cannabis use. Perceived arousal in response to the GSST at T3 was also not associated with cannabis use (yes/no) at T5, nor with CUPIT scores or problematic cannabis use (Table 3). Again, sex did not moderate the relationship between perceived arousal and later cannabis use or problematic cannabis use.

Out of all T3 cannabis users, 113 also used cannabis at T5, and 36 met the definition of problematic cannabis use at T5. Among T3 cannabis users, HPA axis measures at T3 showed no associations with the development of problematic cannabis use at T5 (Table 4). There was a positive association between perceived arousal in anticipation of the stressor and problematic cannabis use at T5 in T3 cannabis users (Table 4).

**Table 1:** Descriptives

| | N | mean | SD |
|---|---|---|---|
| Participants GSST (% male) | 715 (49.1%) | | |
| Age at GSST (T3) | 715 | 16.1 | 0.6 |
| Age at T5 | 715 | 22.2 | 0.6 |
| HPA axis activity (nmol/l) | | | |
| Baseline levels (morning) | 715 | 8.0 | 5.8 |
| CAR | 715 | 5.7 | 7.1 |
| Anticipation | 715 | 5.3 | 4.3 |
| AUCincr | 715 | 63.8 | 101.9 |
| Recovery | 715 | 1.3 | 2.3 |
| Perceived arousal | | | |
| Baseline | 715 | 2.8 | 2.8 |
| Anticipation | 715 | 2.7 | 1.5 |
| Reactivity | 715 | 4.2 | 1.9 |
| Recovery | 715 | 1.8 | 2.0 |
| Substance use | | | |
| Cannabis (past year) at T3 | 189 (26.4%) | | |
| Cannabis (past year) at T5 | 258 (36.1%) | | |
| CUPIT total score at T5 | 232 | 13.8 | 14.0 |
| Problematic use at T5 | 54 (7.6%) | | |

Abbreviations: GSST, Groningen social stress test; HPA, Hypothalamus-pituitary-adrenal; CAR, cortisol awakening response; CUPIT, Cannabis Use Problems Identification Test
Problematic use was defined as CUPIT scores of 20 or higher.
The discrepancy between the number of cannabis users at T5 and those with a CUPIT scores resulted from multiple imputation of the two different variables.

**Table 2a:** Associations between HPA axis measures and cannabis use at T5.
Models adjusted for sex and age at T3.

| **Cannabis use at T5** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Cannabis use (yes/no)** | | | **CUPIT score** | | | **Problematic use** | | |
| | **OR** | **95% CI** | **p** | **β** | **95% CI** | **p** | **OR** | **95% CI** | **P** |
| Morning levels | 0.88 | 0.69; 1.13 | 0.32 | 0.32 | -2.14; 2.77 | 0.80 | 1.14 | 0.68; 1.93 | 0.62 |
| CAR | 0.99 | 0.78; 1.26 | 0.94 | -0.10 | -2.61; 2.41 | 0.94 | 1.27 | 0.73; 2.18 | 0.40 |
| Anticipation | 0.95 | 0.79; 1.15 | 0.61 | -0.38 | -2.15; 1.40 | 0.68 | 0.77 | 0.44; 1.33 | 0.34 |
| Reactivity | 0.92 | 0.74; 1.14 | 0.45 | -1.61 | -3.77; 0.55 | 0.14 | 0.56 | 0.30; 1.05 | 0.07 |
| Recovery | 1.07 | 0.87; 1.34 | 0.52 | 0.52 | -1.67; 2.71 | 0.64 | 1.13 | 0.62; 2.06 | 0.68 |

Abbreviations: HPA, Hypothalamus-pituitary-adrenal; CAR, cortisol awakening response; OR, odds ratio; CI, confidence interval; CUPIT, Cannabis Use Problems Identification Test; β= regression coefficient.
Problematic use was defined as CUPIT scores of 20 or higher.
Adolescents taking medication that affects cortisol levels were excluded from these analyses.

**Table 2b:** Associations between HPA axis measures and cannabis use at T5.
Models adjusted for sex, age, socioeconomic status, externalizing and internalizing problems, alcohol use, smoking and cannabis use at T3, use of oral contraceptives.

| Cannabis use at T5 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Cannabis use (yes/no) | | | CUPIT score | | | Problematic use | | |
| | OR | 95% CI | p | β | 95% CI | p | OR | 95% CI | P |
| Morning levels | 0.92 | 0.71; 1.19 | 0.53 | 0.70 | -1.75; 3.14 | 0.58 | 1.27 | 0.73; 2.22 | 0.40 |
| CAR | 0.99 | 0.77; 1.28 | 0.96 | -0.11 | -2.65; 2.43 | 0.93 | 1.33 | 0.72; 2.45 | 0.36 |
| Anticipation | 0.93 | 0.76; 1.13 | 0.46 | -0.56 | -2.32; 1.19 | 0.53 | 0.72 | 0.40; 1.30 | 0.27 |
| Reactivity | 0.99 | 0.79; 1.24 | 0.93 | -1.39 | -3.53; 0.74 | 0.20 | 0.55 | 0.29; 1.07 | 0.08 |
| Recovery | 1.05 | 0.85; 1.31 | 0.66 | 0.59 | -1.56; 2.74 | 0.59 | 1.14 | 0.64; 2.01 | 0.67 |

Abbreviations: HPA, Hypothalamus-pituitary-adrenal; CAR, cortisol awakening response; OR, odds ratio; CI, confidence interval; CUPIT, Cannabis Use Problems Identification Test; β= regression coefficient.
Problematic use was defined as CUPIT scores of 20 or higher.
Adolescents taking medication that affects cortisol levels were excluded from these analyses.

**Table 3a:** Associations between perceived arousal measures and any cannabis use at T5.
Models adjusted for sex and age at T3.

| Cannabis use at T5 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Cannabis use (yes/no) | | | CUPIT score | | | Problematic use | | |
| | OR | 95% CI | P | β | 95% CI | p | OR | 95% CI | p |
| Perceived arousal | | | | | | | | | |
| Baseline | 1.02 | 0.85; 1.21 | 0.87 | -0.93 | -2.58; 0.72 | 0.15 | 0.85 | 0.58; 1.24 | 0.39 |
| Anticipation | 0.94 | 0.77; 1.15 | 0.53 | 1.50 | -0.33; 3.32 | 0.27 | 1.16 | 0.79; 1.69 | 0.45 |
| Reactivity | 1.00 | 0.76; 1.31 | 0.99 | -0.68 | -3.14; 1.79 | 0.11 | 0.94 | 0.58; 1.51 | 0.78 |
| Recovery | 0.97 | 0.75; 1.26 | 0.83 | -1.14 | -3.52; 1.24 | 0.59 | 0.76 | 0.47; 1.24 | 0.27 |

Abbreviations: OR, odds ratio; CI, confidence interval; CUPIT, Cannabis Use Problems Identification Test, β= regression coefficient.
Problematic use was defined as CUPIT scores of 20 or higher.

## Discussion

In this study we tested whether physiological stress reactivity and perceived arousal at age 16 were associated with problematic cannabis use at age 22. Although we did not find a relationship between HPA axis reactivity and later cannabis use, we found that in adolescents who already used cannabis at age 16, higher perceived arousal in anticipation of a stressor was associated with the development of problematic cannabis use by age 22.

According to the novelty-seeking theory, adolescents who have a blunted stress reactivity and low levels of physiological arousal, would be more inclined to use cannabis in order to retain a higher level of arousal [37,38]. Studies have confirmed

**Table 3b:** Associations between perceived arousal measures and any cannabis use at T5. Models adjusted for sex, age, socio-economic status, externalizing and internalizing problems, alcohol use, smoking and cannabis use at T3.

| Cannabis use at T5 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Cannabis use (yes/no) | | | CUPIT score | | | Problematic use | | |
| | OR | 95% CI | p | β | 95% CI | p | OR | 95% CI | p |
| Perceived arousal | | | | | | | | | |
| Baseline | 0.93 | 0.76; 1.15 | 0.52 | -1.56 | -3.41; 0.28 | 0.10 | 0.67 | 0.42; 1.09 | 0.10 |
| Anticipation | 0.98 | 0.79; 1.21 | 0.84 | 1.73 | -0.06; 3.51 | 0.06 | 1.23 | 0.82; 1.84 | 0.32 |
| Reactivity | 1.00 | 0.75; 1.34 | 0.99 | -1.23 | -3.72; 1.26 | 0.33 | 0.79 | 0.46; 1.35 | 0.38 |
| Recovery | 1.03 | 0.78; 1.35 | 0.85 | -0.54 | -2.88; 1.80 | 0.65 | 0.85 | 0.50; 1.43 | 0.54 |

Abbreviations: OR, odds ratio; CI, confidence interval; CUPIT, Cannabis Use Problems Identification Test, β= regression coefficient.
Problematic use was defined as CUPIT scores of 20 or higher.

**Table 4:** HPA axis and perceived arousal measures and the development of problematic cannabis use in T3 cannabis users.

| | Problematic use | | |
|---|---|---|---|
| | OR | 95% CI | P |
| HPA axis measures | | | |
| Morning levels | 1.57 | 0.65; 3.76 | 0.32 |
| CAR | 1.46 | 0.60; 3.55 | 0.40 |
| Anticipation | 0.47 | 0.14; 1.54 | 0.21 |
| Reactivity | 0.37 | 0.11; 1.27 | 0.12 |
| Recovery | 0.95 | 0.32; 2.78 | 0.92 |
| Perceived arousal measures | | | |
| Baseline | 0.65 | 0.32; 1.33 | 0.24 |
| Anticipation | 2.36 | 1.01; 5.52 | 0.048 |
| Reactivity | 0.60 | 0.24; 1.53 | 0.29 |
| Recovery | 0.96 | 0.41; 2.24 | 0.92 |

Two models (one for HPA axis measures and one for perceived arousal measures) adjusted for sex, age, socio-economic status, externalizing and internalizing problems, alcohol use and smoking.
Abbreviations: HPA, Hypothalamus-pituitary-adrenal; CAR, cortisol awakening response; OR, odds ratio; CI, confidence interval.
Problematic use was defined as CUPIT scores of 20 or higher.

this by showing that lower HPA axis reactivity was associated with earlier onset of cannabis use and with current cannabis use in adolescents [21,22]. However, no studies so far have investigated whether the same predictive power is found with respect to the development of problematic cannabis use (both in the general population, and

among cannabis users only). Our study is the first that addresses the relationship between stress responses and problematic cannabis use in a prospective manner in a general population of adolescents. Results clearly revealed no effect of stress responses here. This lack of associations between HPA-axis measures and problematic cannabis use six years later was unexpected. There was also no association between HPA axis reactivity and overall, non-problematic, future cannabis use in our sample, even though a previous study did find a cross-sectional association between blunted HPA axis reactivity and non-problematic cannabis use in the same sample [22]. It might therefore be that this association is only apparent when measured cross-sectionally and that a six-year gap between HPA axis and cannabis use measurements might be too long to measure associations between the two. While males are more prone to using cannabis and are different in their stress responses than females [39,40], we did not find any moderating effect of sex on the relationship between stress responses and cannabis use. Neither did we find a relationship between stress reactivity and future problematic cannabis use in current cannabis users. It might be that the relationship between stress reactivity and the development of problematic cannabis use is moderated by other factors, such as family history of externalizing problems or substance use, genetic factors, temperament or stressful life events [41].



In T3 cannabis users, only high anticipatory perceived arousal predicted the development of problematic cannabis use. This was in contrast with our hypothesis that low perceived arousal might increase the risk of using cannabis and developing problematic cannabis use in adolescence. The finding could be explained with the tension reduction model, which states that substances are often used to decrease negative affect [9]. Various studies in different populations have reported that relaxation and stress-coping motives are one of the reasons for cannabis use [42,43], especially among heavy users [44]. Studies have also reported increased cannabis use during times of stress [45]. Another study among frequent cannabis users showed that acute negative stressful events and coping motives predicted first time onset of cannabis dependence [46]. Individuals who have a heightened perception of stress may feel more inclined to use substances to relieve the tension that arises when they face a stressor. Hence, high perceived arousal in anticipation of an upcoming stressor may contribute to an increased risk for problematic cannabis use, especially among early cannabis users. Studies that focus on the psychological stress response and later substance use are still very rare, we are not aware of any other study on this subject. Therefore, our results should be interpreted with caution and replication studies are required.

Interestingly, previous studies investigating the initiation of cannabis use have found in general that a blunted stress response is associated with enhanced risk for the initiation of cannabis use [17,22,47]. This finding has also been reported for other substances such as alcohol and tobacco [48], and seems in contrast with the finding that an enhanced stress response is associated with substance use [42]. The present study might shed some light on these conflicting notions. While a blunted stress

response seems relevant for the initiation of cannabis use, we show that an increased perceived stress response might be relevant for continued and more problematic cannabis use. The present findings suggest that the role of stress in cannabis use (and probably the use of other substances) is not straightforward but depends on the stage of substance use. The role of stress in the first stages of drug use might even be the opposite of that in later, more problematic stages of use. In line with this, previous studies have suggested that different predictors are important in the transition from irregular to frequent cannabis use versus the transition from frequent cannabis use to dependence [46,49].



One strength of the study, is that we address a stage of use that is not often studied. Most studies investigate the role of stress in cannabis use initiation or patients with a cannabis use disorder. The present study sheds some light on the role of stress in the transition from cannabis use to problematic use. The use of a prospective longitudinal design made it possible to study whether stress responses were indeed a precursor of problematic cannabis use. The measurement of multiple covariates and the large sample size allowed us to adjust for possible confounders and to test the moderating effect of sex. Nevertheless, there are several limitations to this study. First, the stress reactivity parameters were only measured once. Regulation of the HPA axis and perceived arousal change over time and in relation to substance use and it is possible that we did not find any relationship due to the six-year gap between the two measurements. Despite the large sample size, the number of adolescents with high scores on the CUPIT was small and to study problematic cannabis use it would be favorable to choose a high risk sample. The cut-off of 20 or higher on the CUPIT questionnaire was adapted from Bashford et al. (2010) but it has not been validated for the Netherlands and it might be that adolescents with a lower score also qualify as problematic users. In addition, we cannot be sure whether some of the T3 cannabis users also qualified as problematic cannabis users. However, results did not change when we excluded adolescents who had used cannabis more than 40 times in the past year from the analyses. Hence, this probably did not influence our results.

Although the HPA axis has been implicated in substance use and addiction, we could not confirm a relationship between HPA axis reactivity and problematic cannabis use in this longitudinal study. The relationship between stress reactivity and substance use is not straightforward and different mechanisms could be involved in different populations of adolescents, as well as in different stages of substance use. Future research could test moderating effects of other variables such as negative stressful life events, genetic polymorphisms or temperament on this relationship. Our results suggest a role of perceived arousal in the development of problematic cannabis use in adolescents, and presents an interesting starting point to further study which adolescents develop problematic cannabis use, and to further investigate the role of stress in problematic cannabis - and probably other drug - use.

## References


1. Copeland J, Swift W (2009) Cannabis use disorder: epidemiology and management. Int Rev Psychiatry 21: 96-103.
2. Gruber AJ, Pope HG, Jr. (2002) Marijuana use among adolescents. Pediatr Clin North Am 49: 389-413.
3. De Looze M, Van Dorsselaer S, De Roos S, Verdurmen J, Stevens G, et al. (2014) HBSC 2013: gezondheid, welzijn en opvoeding van jongeren in Nederland. Universiteit Utrecht Utrecht.
4. Hibell B, Guttermsson U, Ahlström S, Balakireva O, Bjarnason T, et al. (2012) The 2011 ESPAD report: substance use among students in 35 European countries. . CAN, Stockholm.
5. de Graaf R, ten Have M, van Gool C, van Dorsselaer S (2012) Prevalence of mental disorders and trends from 1996 to 2009. Results from the Netherlands Mental Health Survey and Incidence Study-2. Soc Psychiatry Psychiatr Epidemiol 47: 203-213.
6. Meier MH, Caspi A, Ambler A, Harrington H, Houts R, et al. (2012) Persistent cannabis users show neuropsychological decline from childhood to midlife. Proc Natl Acad Sci U S A 109: E2657-2664.
7. Medina KL, Hanson KL, Schweinsburg AD, Cohen-Zion M, Nagel BJ, et al. (2007) Neuropsychological functioning in adolescent marijuana users: subtle deficits detectable after a month of abstinence. J Int Neuropsychol Soc 13: 807-820.
8. Jacobus J, Bava S, Cohen-Zion M, Mahmood O, Tapert SF (2009) Functional consequences of marijuana use in adolescents. Pharmacol Biochem Behav 92: 559-565.
9. Conger JJ (1956) Alcoholism: theory, problem and challenge. II. Reinforcement theory and the dynamics of alcoholism. Q J Stud Alcohol 17: 296-305.
10. Lewinsohn PM, Rohde P, Brown RA (1999) Level of current and past adolescent cigarette smoking as predictors of future substance use disorders in young adulthood. Addiction 94: 913-921.
11. Bardo MT, Donohew RL, Harrington NG (1996) Psychobiology of novelty seeking and drug seeking behavior. Behav Brain Res 77: 23-43.
12. Korhonen T, van Leeuwen AP, Reijneveld SA, Ormel J, Verhulst FC, et al. (2010) Externalizing behavior problems and cigarette smoking as predictors of cannabis use: the TRAILS Study. J Am Acad Child Adolesc Psychiatry 49: 61-69.
13. Windle M, Wiesner M (2004) Trajectories of marijuana use from adolescence to young adulthood: Predictors and outcomes. Development and Psychopathology 16: 1007-1027.
14. Stephenson MT, Helme DW (2006) Authoritative parenting and sensation seeking as predictors of adolescent cigarette and marijuana use. J Drug Educ 36: 247-270.
15. Creemers HE, Korhonen T, Kaprio J, Vollebergh WA, Ormel J, et al. (2009) The role of temperament in the relationship between early onset of tobacco and cannabis use: the TRAILS study. Drug Alcohol Depend 104: 113-118.
16. Moss HB, Vanyukov M, Yao JK, Kirillova GP (1999) Salivary cortisol responses in prepubertal boys: the effects of parental substance abuse and association with drug use behavior during adolescence. Biol Psychiatry 45: 1293-1299.
17. Moss HB, Vanyukov MM, Martin CS (1995) Salivary cortisol responses and the risk for substance abuse in prepubertal boys. Biol Psychiatry 38: 547-555.
18. Murphy LL, Munoz RM, Adrian BA, Villanua MA (1998) Function of cannabinoid receptors in the neuroendocrine regulation of hormone secretion. Neurobiol Dis 5: 432-446.

19. Monteleone P, Di Filippo C, Fabrazzo M, Milano W, Martiadis V, et al. (2014) Flattened cortisol awakening response in chronic patients with schizophrenia onset after cannabis exposure. Psychiatry Res 215: 263-267.

20. Steptoe A, Ussher M (2006) Smoking, cortisol and nicotine. International Journal of Psychophysiology 59: 228-235.

21. Huizink AC, Ferdinand RF, Ormel J, Verhulst FC (2006) Hypothalamic-pituitary-adrenal axis activity and early onset of cannabis use. Addiction 101: 1581-1588.

22. van Leeuwen AP, Creemers HE, Greaves-Lord K, Verhulst FC, Ormel J, et al. (2011) Hypothalamic-pituitary-adrenal axis reactivity to social stress and adolescent cannabis use: the TRAILS study. Addiction 106: 1484-1492.

23. Kirschbaum C, Pirke KM, Hellhammer DH (1993) The 'Trier Social Stress Test'--a tool for investigating psychobiological stress responses in a laboratory setting. Neuropsychobiology 28: 76-81.

24. de Winter AF, Oldehinkel AJ, Veenstra R, Brunnekreef JA, Verhulst FC, et al. (2005) Evaluation of non-response bias in mental health determinants and outcomes in a large sample of pre-adolescents. Eur J Epidemiol 20: 173-181.

25. Nederhof E, Jorg F, Raven D, Veenstra R, Verhulst FC, et al. (2012) Benefits of extensive recruitment effort persist during follow-ups and are consistent across age group and survey method. The TRAILS study. BMC Med Res Methodol 12: 93.

26. Booij SH, Bouma EM, de Jonge P, Ormel J, Oldehinkel AJ (2013) Chronicity of depressive problems and the cortisol response to psychosocial stress in adolescents: the TRAILS study. Psychoneuroendocrinology 38: 659-666.

27. Bouma EM, Riese H, Ormel J, Verhulst FC, Oldehinkel AJ (2009) Adolescents' cortisol responses to awakening and social stress; effects of gender, menstrual phase and oral contraceptives. The TRAILS study. Psychoneuroendocrinology 34: 884-893.

28. Kudielka BM, Schommer NC, Hellhammer DH, Kirschbaum C (2004) Acute HPA axis responses, heart rate, and mood changes to psychosocial stress (TSST) in humans at different times of day. Psychoneuroendocrinology 29: 983-992.

29. Bashford J, Flett R, Copeland J (2010) The Cannabis Use Problems Identification Test (CUPIT): development, reliability, concurrent and predictive validity among adolescents and adults. Addiction 105: 615-625.

30. Kirschbaum C, Read GF, Hellhammer D (1992) Assessment of hormones and drugs in saliva in biobehavioral research. Seattle: Hogrefe & Huber. 356 p. p.

31. Janssens KA, Oldehinkel AJ, Verhulst FC, Hunfeld JA, Ormel J, et al. (2012) Symptom-specific associations between low cortisol responses and functional somatic symptoms: the TRAILS study. Psychoneuroendocrinology 37: 332-340.

32. Wald FDM, Mellenbergh GJ (1990) De verkorte versie van de Nederlandse vertaling van de Profile of Mood States (POMS). Nederlands Tijdschrift voor de Psychologie, 45, 86-90.

33. Bradley MM, Lang PJ (1994) Measuring emotion: the Self-Assessment Manikin and the Semantic Differential. J Behav Ther Exp Psychiatry 25: 49-59.

34. Mehrabian A, Russell JA (1974) The basic emotional impact of environments. Percept Mot Skills 38: 283-301.

35. Achenbach TM (1991) Manual for the youth self-report and 1991 profile. . Burlington: University of Vermont Department of Psychiatry.

36. Heron J, Hickman M, Macleod J, Munafo MR (2011) Characterizing Patterns of Smoking Initiation in Adolescence: Comparison of Methods for Dealing With Missing Data. Nicotine & Tobacco Research 13: 1266-1275.

37. Roberti JW (2004) A review of behavioral and biological correlates of sensation seeking. Journal of Research in Personality 38: 256-279.

38. Marschall-Levesque S, Castellanos-Ryan N, Vitaro F, Seguin JR (2014) Moderators of the association between peer and target adolescent substance use. Addictive Behaviors 39: 48-70.

39. Seeman TE, Singer B, Wilkinson CW, McEwen B (2001) Gender differences in age-related changes in HPA axis reactivity. Psychoneuroendocrinology 26: 225-240.

40. Kudielka BM, Buske-Kirschbaum A, Hellhammer DH, Kirschbaum C (2004) HPA axis responses to laboratory psychosocial stress in healthy elderly adults, younger adults, and children: impact of age and gender. Psychoneuroendocrinology 29: 83-98.

41. Verweij KJ, Zietsch BP, Lynskey MT, Medland SE, Neale MC, et al. (2010) Genetic and environmental influences on cannabis use initiation and problematic use: a meta-analysis of twin studies. Addiction 105: 417-430.

42. Hyman SM, Sinha R (2009) Stress-related factors in cannabis use and misuse: implications for prevention and treatment. J Subst Abuse Treat 36: 400-413.

43. Hathaway AD, Callaghan RC, Macdonald S, Erickson PG (2009) Cannabis dependence as a primary drug use-related problem: the case for harm reduction-oriented treatment options. Subst Use Misuse 44: 990-1008.

44. Johnston LD, Omalley PM (1986) Why Do the Nations Students Use Drugs and Alcohol - Self-Reported Reasons from 9 National Surveys. Journal of Drug Issues 16: 29-66.

45. Kaplan HB, Martin SS, Johnson RJ, Robbins CA (1986) Escalation of marijuana use: application of a general theory of deviant behavior. J Health Soc Behav 27: 44-61.

46. van der Pol P, Liebregts N, de Graaf R, Korf DJ, van den Brink W, et al. (2013) Predicting the transition from frequent cannabis use to cannabis dependence: A three-year prospective study. Drug and Alcohol Dependence 133: 352-359.

47. Dawes MA, Dorn LD, Moss HB, Yao JK, Kirisci L, et al. (1999) Hormonal and behavioral homeostasis in boys at risk for substance abuse. Drug Alcohol Depend 55: 165-176.

48. Lovallo WR (2006) Cortisol secretion patterns in addiction and addiction risk. International Journal of Psychophysiology 59: 195-202.

49. van der Pol P, Liebregts N, de Graaf R, Ten Have M, Korf DJ, et al. (2013) Mental health differences between frequent cannabis users with and without dependence and the general population. Addiction 108: 1459-1469.

# chapter 7

## Catechol-O-methyltransferase gene methylation and substance use in adolescents: The TRAILS study.

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## Abstract

Substance use often starts in adolescence and poses a major problem for society and individual health. The dopamine system plays a role in substance use, and catechol-O-methyltransferase (COMT) is an important enzyme that degrades dopamine. The Val$^{108/158}$Met polymorphism modulates COMT activity and thus dopamine levels, and has been linked to substance use. *COMT* gene methylation, on the other hand, may affect expression and thus indirectly COMT activity. We investigated whether methylation of the *COMT* gene was associated with adolescents' substance use. Furthermore, we explored whether the *COMT* Val$^{108/158}$Met polymorphism interacts with *COMT* gene methylation in the association with substance use. In 463 adolescents (mean age=16, 50.8% girls), substance use (cigarette smoking, alcohol and cannabis use) was assessed with self-report questionnaires. From blood samples, *COMT* Val$^{108/158}$Met genotype and methylation rates of membrane bound (MB) and soluble (S) *COMT* promoters were assessed. *MB-COMT* promoter methylation was associated with non-daily smoking (OR=1.82, p=0.03), but not with daily smoking (OR=1.20, p=0.34), *MB-COMT* promoter methylation was not associated with alcohol use. Adolescents with the Met/Met genotype and high rates of *MB-COMT* promoter methylation were less likely to be high frequent cannabis users than adolescents with the Val/Val or Val/Met genotype. *S-COMT* promoter methylation was not associated with substance use. These results indicate that there is an association between substance use and *COMT* gene methylation. Although this association is complex, combining genetic and epigenetic variation of the *COMT* gene may be helpful in further elucidating the influence of the dopamine system on substance use in adolescence.

## Introduction

Substance use (i.e. alcohol, cigarettes or cannabis) often starts in adolescence. Prolonged use can lead to poor health, and detrimental social and economic outcomes [1]. The dopaminergic reward system plays an important role in substance use and addiction [2]. Frequent substance use is associated with altered dopamine levels in the brain reward system [3]. Catechol-O-methyltransferase (COMT) degrades dopamine, and variations in COMT expression and activity could modify reward system functioning, thereby influencing vulnerability to substance use.

The *COMT* gene (chr:22, q11.21, [4]) encodes two different protein isoforms, each with its own promoter [5]: the membrane-bound isoform (*MB-COMT*, 271 amino acids), and the soluble isoform (*S-COMT*, 221 amino acids). The functional $Val^{108/158}Met$ single nucleotide polymorphism (SNP) in the *COMT* gene, rs4680, has been associated with altered COMT activity [6]. The Val/Val genotype results in a three to fourfold increase in COMT activity and was more prevalent in substance users [7-11], albeit not consistently [12]. These findings indicate a higher COMT activity, hence faster dopamine degradation, in substance users, which arguably is associated with a drive for constant activation of the reward system.

While *COMT* genotypes influence COMT activity, epigenetic modifications (e.g. DNA methylation) of the *COMT* gene may affect gene expression. Indeed, increased *COMT gene* methylation was associated with decreased gene expression [13,14], but very little is known about the association between *COMT gene* methylation and substance use. In the only general population study we know of, nicotine dependence was related to higher *MB-COMT* promoter methylation, suggesting lower *COMT* gene activity and thus less dopamine degradation in smokers [15]. In schizophrenia patients, alcohol use was associated with increased *MB-COMT* promoter methylation [13]. While studies on genetic variation suggest COMT hyperactivity in substance users, these first epigenetic results indicate lower *COMT* gene activity in substance users. No studies have yet investigated the relationship between cannabis use and *COMT* gene methylation.

In this study we investigated the association between substance use (i.e., cigarettes, alcohol and cannabis) and *COMT* gene methylation in the *MB-COMT* promoter (previously studied by [15]), as well as the *S-COMT* promoter (not studied previously). We used DNA from a large general population sample of adolescents (14-18 years). Given the lack of studies so far, we carefully hypothesized that *COMT* gene methylation will not only be associated with tobacco and alcohol use, but also with cannabis use. Given the seemingly contradictory findings on *COMT* genotype and *COMT* gene methylation (increased activity vs lower expression of COMT in substance users), an interplay between the two may be present, with indirect oppositional effects on dopamine levels. Therefore, we explored whether the association between *COMT* gene methylation and substance use depended on the *COMT* $Val^{108/158}Met$ polymorphism.

## Material and Methods

### Subjects

This study was part of the TRacking Adolescents' Individual Lives Survey (TRAILS), a prospective population study in which Dutch preadolescents (N=2230) are followed into adulthood. Assessment waves, involving interviews, biological measures and validated questionnaires, are conducted biennially or triennially, and five assessment waves have been completed so far. The present study involves data collected during the third assessment wave, which took place from September 2005 to December 2007 (N=1816, mean age 16.3 years, SD=0.71). Written consent was obtained from each subject and their parents at every assessment wave. The study was approved by the Dutch Central Medical Ethics Committee (CCMO) and all subjects received compensation for their participation. A detailed description of sampling and methods can be found in [16] and [17]. In short, the assessment at T3 included an extensive experimental session, in which 715 adolescents participated (focus sample, response rate 96.1%). Adolescents with a higher risk of mental health problems had a greater chance of being selected for the experimental session. Risk was defined based on T1 measures of temperament (high frustration and fearfulness, low effortful control), lifetime parental psychopathology, and living in a single-parent family. In total 66.0% of the focus sample had at least one of the above described risk factors; the remaining 34.0% were selected randomly from the 'low-risk' TRAILS participants. Although 'high-risk' adolescents were slightly oversampled, the sample included the total range of mental health problems present in a community population of adolescents. T3 also involved a blood draw. Selection for methylation analyses (N=475) was based on the participation in the extensive experimental session, availability of a blood sample with sufficient DNA concentration, Dutch ethnicity, and we randomly excluded one of each sibling pair. This selection of 475 adolescents did not differ significantly ($p>.05$) from the TRAILS focus sample (N=715) with regard to sex, socioeconomic status and age. Following drop-out after methylation analyses (for further explanation see section on methylation analyses), we obtained *MB-COMT* promoter methylation rates for 458 subjects, and *S-COMT* promoter methylation rates for 463 subjects.

### Substance use

Substance use was assessed with a self-report questionnaire at T3, which was filled out at school or at the subjects' home. Confidentiality of the study was important and adolescents were reassured that their parents or teachers would not have access to the information they provided. Smoking was assessed with the question: "How many cigarettes did you smoke in the past 4 weeks?". Adolescents who had not smoked in the past 4 weeks were categorized as non-smokers. Adolescents who had smoked less than one cigarette a day in the past 4 weeks were categorized as non-daily smokers and those who had smoked one or more cigarettes per day as daily smokers [18]. Cannabis use was assessed with the question: "How many times have you used weed

(marijuana) or hash in the past 4 weeks?". Adolescents who had not used cannabis in the past 4 weeks were categorized as non-users. Adolescents who had used up to four times were categorized as low-frequent users and those who used more than four times as high-frequent users [19]. Alcohol use was assessed with the question: "How many times have you had alcohol in the past 4 weeks? By this, we mean the number of occasions, like going to a party, going out, or an evening at home". Adolescents who reported that they had not drunk alcohol in the past 4 weeks were categorized as abstainers. Adolescents who reported drinking were categorized into two groups: those who had drunk alcohol up to 9 times were defined as low-frequent users and those who had drunk alcohol 10 times or more were defined as high-frequent users [19].



## DNA methylation

*Analysis.* DNA was extracted from whole-blood samples using a manual salting-out procedure [20]. Primer sets described previously [15,21] were used to analyze regions of the CpG islands (regions containing high frequencies of CpG sites) in the *S-COMT* (S2, Chr 22: 19949993-19950393 [21]) and *MB-COMT* (Chr 22: 19928950-19929359 [15]) promoters (Figure 1).

DNA methylation rates were analyzed using the EpiTYPER method from Sequenom. Bisulfite conversion was followed by PCR amplification, reverse transcription and base-specific cleavage. Fragments were analyzed on a mass spectrometer (Sequenom EpiTYPER, San Diego, CA, USA). Bisulfite conversion of DNA was performed using EZ-96 DNA Methylation Kit (Shallow) (Zymo Research, CA, USA), according to manufacturers' protocol. PCR, reverse transcription, cleavage and mass spectrometry were performed in triplicate, according to EpiTYPER protocol. The mass signal patterns generated were translated to quantitative methylation rates for different CpG-units by the MassARRAY EpiTYPER analyzer software from Sequenom. (v1.0, build1.0.6.88 Sequenom, Inc, San Diego, USA). Fragments with CpG dinucleotides are referred to as CpG units. One CpG unit can contain one or more CpG dinucleotides. CpG units with a mass outside the range of the mass spectrometer, or with overlap in mass of another CpG unit, could not be analyzed (*MB-COMT*: 7 CpG units, *S-COMT*: 6 CpG units).

*Data cleaning procedures.* All samples were analyzed in triplicate and for each CpG unit, methylation rates of the triplicates were averaged [22]. Samples with a standard deviation of ≥10% between replicates were removed for analysis. CpG units with ≥25% missing values were not included in the analyses (two CpG units, CpGU2 and CpGU3, in the *S-COMT* promoter and one CpG unit, CpGU16, in the *MB-COMT* promoter). We accounted for mass-change in CpG units by SNPs (only when minor allele frequency >5%) by removing CpG units containing SNPs from analyses (1 CpG unit (*S-COMT* promoter, CpGU7)), and by removing units with the same mass as non-CpG units containing SNPs or other CpG units containing SNPs

(none in our sample)). In total, eleven CpG units were available for the *MB-COMT* promoter region and five CpG units were available for the *S-COMT* promoter region.

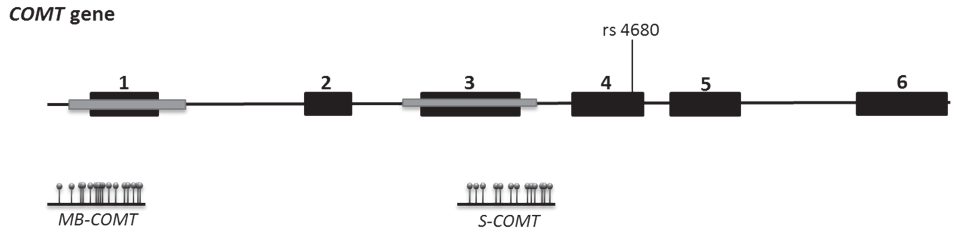




**Figure 1.** Schematic representation of the COMT gene (location: 22q11.21). Black numbered boxes represent exons 1-6 and the approximate position of the COMT Val108/158Met polymorphism (rs4680, Guanine→Adenine, substituting the amino acid Valine (Val) for Methionine (Met) at position 108/158 in the amino acid chain for S-COMT and MB-COMT respectively) in exon 4 is shown. Grey boxes represent the CpG islands in the MB-COMT and S-COMT promoters in exon 1 and 3 respectively. The DNA fragments of MB-COMT and S-COMT promoters used for methylation analyses are shown in relative position to the CpG islands. Adapted from [21].

## Genotyping

The *COMT* Val$^{108/158}$Met SNP (rs4680) genotyping was performed on the Illumina BeadStation 500 platform (Illumina Inc., San Diego, CA) using Golden Gate assay and array technology (for details, see [23,24]). Data on the Val$^{108/158}$Met genotype (Val/Val, Val/Met or Met/Met) was available for 1411 of the TRAILS subjects, of whom 452 had complete data on both genotype and methylation rates (Table 1). The lower number available for methylation analyses resulted from a pre-selection of subjects (see above). The genotyping call rate for rs4680 was 100%. A $X^2$ -test confirmed that rs4680 was in Hardy-Weinberg equilibrium (p=.92).

## Statistical analyses

Descriptives were computed and ANOVA was performed to compare S-COMT/MB-COMT promoter methylation rates between different genotypes. We used multinomial logistic regression to study the association between COMT genotype and substance use, using the Met/Met genotype (with the lowest enzyme activity) as the reference category. To avoid loss of power when comparing different substance use categories, we did not limit our sample to individuals with methylation data, but we used the genotype data of the 1411 TRAILS subjects.

Since missing methylation values (1-6% *MB-COMT* promoter, 2-22% *S-COMT* promoter) in higher methylated units affect the average methylation rate, we mean-centered our methylation data for each CpG unit (resulting in a mean methylation of 0, with original standard deviation (SD)) thereby maintaining the individual variation in

CpG-units. We then averaged mean-centered methylation over the CpG units within the *MB-COMT* and *S-COMT* promoter regions. This procedure was used before [22].

To test whether substance use was associated with methylation rates of the *MB-COMT* or *S-COMT* promoter regions, we used multinomial logistic regression analyses. The group of non-substance users was used as reference group in all analyses. In addition, we tested whether the interaction between the *COMT* $Val^{108/158}Met$ genotype and methylation was significantly associated with substance use. If this was the case, analyses were stratified by *COMT* genotype.

As Xu et al. demonstrated CpG-site specific associations of the *MB-COMT* promoter with nicotine dependence (for overlap with CpG units in the current study, see Table S1/Figure S1), we tested whether methylation rates differed between substance use categories for individual CpG units using multinomial logistic regression, with non-substance users as reference category. For these exploratory analyses, we adjusted for multiple testing using the Bonferroni method. The new p-value regarded as significant was 0.0045.

We adjusted all our analyses for age and sex, as age and sex are both related to DNA methylation [25] and substance use [26]. The sample size varied over analyses depending on the number of missing data (see Table 1).



## Results

### Descriptives

Alcohol use was the most commonly reported type of substance use in our study population. Smoking was also frequently reported (Table 1). The ranges of methylation of the different CpG units are shown in Table S2. *S-COMT* promoter methylation was higher in adolescents with the Met/Met genotype ($F(2,449)=4.178$, $p<0.05$, Figure 2).

Val/Val adolescents were less likely to be low-frequent cannabis users (OR=0.57, CI=0.32; 1.01, p=0.06) or high-frequent cannabis users (OR=0.45, CI=0.21; 0.96, p=0.04) than non-users, compared to adolescents with the Met/Met genotype. The Val/Met genotype did not have significantly different odds of cannabis use compared to the Val/Val genotype. There was no significant association between *COMT* genotype and smoking or alcohol use.

### Substance use and *COMT* gene methylation

As shown in Table 2, *MB-COMT* promoter methylation was associated with non-daily smoking, but not with daily smoking. *MB-COMT* or *S-COMT* promoter methylation were not associated with cannabis use or alcohol use (Table 2). Due to differences in *S-COMT* promoter methylation according to *COMT* genotype (described above, Figure 2) we added genotype as a covariate in the analyses with *S-COMT* promoter methylation. This did not change the relationships between *S-COMT* promoter methylation and substance use.

**Table 1:** Descriptives

| | N (%) | mean (SD) |
|---|---|---|
| Age | 463 (100%) | 16.1 (0.6) |
| Girls | 235 (50.8%) | |
| Smoking (n=458) | | |
| None | 332 (72.5%) | |
| not daily | 34 (7.4%) | |
| Daily | 92 (20.1%) | |
| Cannabis use (n=452) | | |
| None | 391 (86.5%) | |
| Low-frequent | 34 (7.5%) | |
| High-frequent | 27 (6.0%) | |
| Alcohol use (n=449) | | |
| None | 89 (19.8%) | |
| Low-frequent | 303 (67.5%) | |
| High-frequent | 57 (12.3%) | |
| Val/Met polymorphism (n=452) | | |
| Met/Met | 141 (31.2%) | |
| Val/Met | 222 (49.1%) | |
| Val/Val | 89 (19.7%) | |



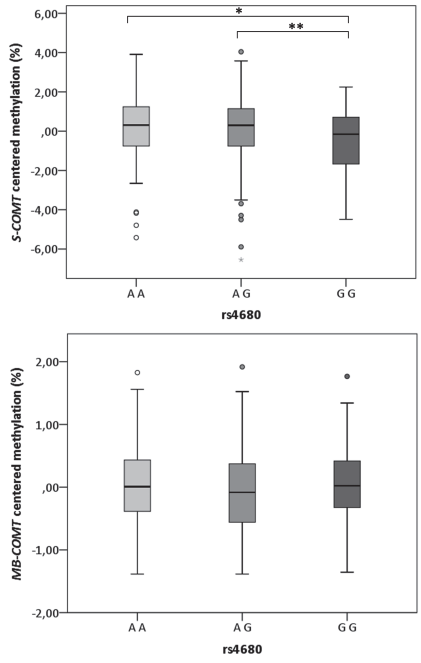


**Figure 2.** Centered methylation rates of the *S-COMT* and *MB-COMT* promoter for each genotype (A=Met, G=Val, bars: p25-p75). *p<0.05, **p<0.01

**Table 2:** Associations between methylation of the COMT gene and substance use

| | OR | 95% CI | p | OR | 95% CI | p |
|---|---|---|---|---|---|---|
| Smoking | | Non daily | | | Daily | |
| MB-COMT promoter methylation | 1.82 | 1.07; 3.09 | 0.03 | 1.20 | 0.83; 1.73 | 0.34 |
| S-COMT promoter methylation | 1.04 | 0.83; 1.30 | 0.76 | 1.09 | 0.94; 1.27 | 0.27 |
| Cannabis | | Low frequent | | | High frequent | |
| MB-COMT promoter methylation | 0.82 | 0.47; 1.42 | 0.48 | 0.70 | 0.38; 1.31 | 0.27 |
| S-COMT promoter methylation | 0.96 | 0.77; 1.19 | 0.68 | 1.06 | 0.82; 1.36 | 0.67 |
| Alcohol | | Low frequent | | | High frequent | |
| MB-COMT promoter methylation | 0.91 | 0.63; 1.33 | 0.63 | 1.00 | 0.59; 1.67 | 0.99 |
| S-COMT promoter methylation | 0.99 | 0.85; 1.15 | 0.85 | 0.90 | 0.73; 1.10 | 0.30 |

Abbreviations: OR, odds ratio; CI, confidence interval.
Reference categories: No smoking, no cannabis use and no alcohol use
Adjusted for sex and age.



In the individual CpG unit analyses (Figure 3), we found that *MB-COMT* promoter methylation in CpG unit 3 (OR=1.17, 95% CI= 1.05;1.30, p=0.004) and CpG unit 9 (OR=1.64, 95% CI= 1.06; 2.52, p=0.03) were associated with non-daily smoking, although only the effect for *MB-COMT*-promoter CpGU3 remained significant after correction for multiple testing. There were no associations between methylation rates of single CpG units and cannabis use. For alcohol use we found that *MB-COMT* promoter methylation was associated with low frequent alcohol use in CpGU5 (OR=0.76, 95% CI= 0.58; 0.995, p=0.046) and in CpGU19 (OR=0.79, 95% CI=0.64; 0.98, p=0.03). These associations were not significant after correction for multiple testing. No effects were found for *S-COMT* promoter methylation.

## Moderation by COMT genotype

When included in our model, the interaction term 'COMT *genotype x* MB-COMT *promoter methylation*' was associated with cannabis use (Table 3). Therefore, we stratified the analyses for methylation and cannabis use by *COMT* Val$^{108/158}$Met genotype. In adolescents with the Met/Met genotype, methylation rates were associated with lower odds of high frequent cannabis use (OR=0.25, 95% CI=0.08; 0.82, p=0.02). The interaction term 'COMT *genotype x* MB-COMT *promoter methylation*' was not associated with smoking or alcohol use and we also did not find any significant association between the interaction term 'COMT *genotype x* S-COMT *promoter methylation*' and substance use.

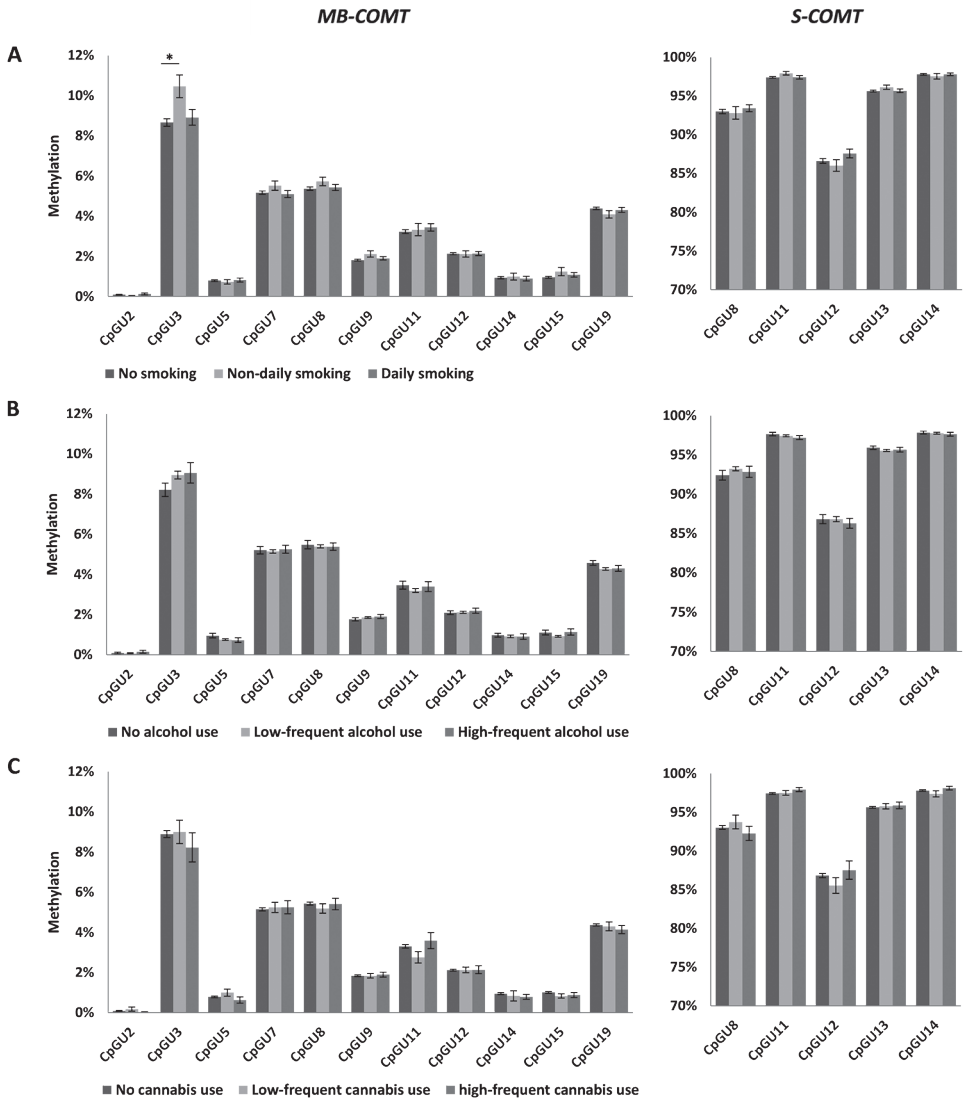


**Figure 3.** Methylation rates of individual CpG Units, divided into substance use categories. Separate graphs were used for the *MB-COMT* promoter (left) and *S-COMT* promoter (right). Mean methylation rates are presented for smoking categories (A), alcohol use categories (B), and for cannabis use categories (C). Error bars represent standard errors. *p<0.0045

**Table 3:** Influence of Val$^{108/158}$Met genotype on association between *MB-COMT* promoter methylation and substance use.

| | OR | 95% CI | p | OR | 95% CI | p |
|---|---|---|---|---|---|---|
| Smoking | | Non daily | | | Daily | |
| Val/Met genotype | 0.61 | 0.34; 1.08 | 0.09 | 0.92 | 0.65; 1.29 | 0.62 |
| *MB-COMT* promoter methylation | 1.10 | 0.10; 12.49 | 0.94 | 1.69 | 0.32; 8.97 | 0.54 |
| Val/Met**MB-COMT* promoter methylation | 1.16 | 0.49; 2.73 | 0.74 | 0.86 | 0.49; 1.51 | 0.61 |
| Cannabis | | Low frequent | | | High frequent | |
| Val/Met genotype | 0.45 | 0.26; 0.79 | 0.01 | 0.60 | 0.33; 1.10 | 0.10 |
| *MB-COMT* promoter methylation | 1.33 | 0.12; 15.07 | 0.82 | 0.07 | 0.01; 0.75 | 0.03 |
| Val/Met**MB-COMT* promoter methylation | 0.83 | 0.34; 2.06 | 0.69 | 2.42 | 1.03; 5.66 | 0.04 |
| Alcohol | | Low frequent | | | High frequent | |
| Val/Met genotype | 0.97 | 0.69; 1.37 | 0.87 | 1.00 | 0.62; 1.62 | 1.00 |
| *MB-COMT* promoter methylation | 1.11 | 0.21; 5.77 | 0.90 | 3.08 | 0.62; 35.51 | 0.34 |
| Val/Met**MB-COMT* promoter methylation | 0.95 | 0.55; 1.64 | 0.84 | 0.66 | 0.30; 1.43 | 0.29 |

Abbreviations: OR, odds ratio; CI, confidence interval.
Reference categories: No smoking, no cannabis use and no alcohol use
Adjusted for sex and age.



## Discussion

This is the first study in which the association between *COMT* gene methylation and adolescents' substance use is analyzed. We found higher methylation rates in non-daily smokers compared to non-smokers and daily smokers. Also, in adolescents homozygous for the Met allele, methylation was associated with lower odds for cannabis use.

Higher rates of *MB-COMT* promoter methylation were associated with non-daily smoking in adolescents. It is difficult to explain why no association with daily smoking was found. We could speculate that specific, currently unknown, regulation mechanisms are at work linking methylation of the *MB-COMT* promoter with non-daily smoking, which represents a more controlled form of smoking in adolescents. In the study by [15], no association between daily smoking and overall *MB-COMT* promoter methylation was found. However, differences in methylation between their daily smokers and controls became apparent when testing individual CpG sites: methylation rates were higher in daily smokers compared to non-smokers at CpG sites -193 and -39, which correspond with CpGU3 and CpGU12 in the current study (Table S1/Figure S1). Interestingly, in the unit specific analyses for smoking, we also found a higher methylation of CpGU3, but specific for non-daily smoking. We did not find differences in methylation of CpGU12 between the smoking groups. It is possible that during adolescence the relationship between methylation and smoking status is different from that later in life. The rate of methylation in the *MB-COMT* promoter

in our study was relatively low compared to the methylation rates reported by [15], possibly due to chronic heavy smoking. Or, as DNA methylation rates increase with age [25], particularly in CpG islands [27], differences in methylation rates may reflect differences in age between the samples (~16 year old adolescents vs ~45 year old adults). Our findings and those from Xu et al show that *COMT* gene methylation is associated with smoking status, but also raise many questions concerning the exact relationship. Since these are the first studies in this area this should not be surprising and obviously further research is necessary to gain insight into smoking habits and *COMT* gene methylation. Longitudinal studies with repeated measures of both methylation and smoking habits will be necessary to further increase our understanding of how both interrelate.



While other studies have found relationships between methylation of genes in the dopamine system and alcohol dependence, e.g., higher rates of methylation in the dopamine transporter gene [28] and methylation of monoamine oxidase-A [29], we did not find a relationship between mean *MB-COMT* or *S-COMT* promoter methylation and alcohol use in adolescents. Neither did we find associations between methylation and alcohol use in our unit-specific analyses. We are not aware of any other study relating methylation of the *COMT* gene to alcohol use in adolescents.

In our study, the Val/Val variant was associated with lower odds of high frequent cannabis use in adolescents. A recent meta-analysis of the association between the *COMT* Val$^{108/158}$Met polymorphism and substance use identified the Val-allele as risk factor for smoking and for cannabis use [12], but the populations studied were highly heterogeneous. Adolescence is a phase in which novelty-seeking, impulsivity and peer behavior may play a major role in the initiation of substance use [30-32]. In line with this theory are findings from studies that have linked the Met/Met variant to increased novelty-seeking, which could drive cannabis use in adolescents [33-35]. Another study associated the Val/Val variant with novelty-seeking [36]. The relationship might be dependent on genetic variations in other genes in addition to the *COMT* polymorphism [37] and might be moderated by personality, stress or other environmental factors.

We found that adolescents with the Met/Met genotype and higher methylation rates had a lower risk for cannabis use. Hence, there seems to be an interaction between the *COMT* Val$^{108/158}$Met polymorphism and *MB-COMT* promoter methylation rates in relation to cannabis use. This is a novel finding which is in line with the anhedonia hypothesis of substance use [38].The combination of the low enzyme activity (Met/Met genotype) and reduced expression of the enzyme (higher methylation rates) might result in higher levels of dopamine through diminished dopamine degradation. It is known that low brain dopamine levels result in an under-active reward system, accompanied by anhedonia. Substance use could be explained as an attempt to alleviate this unfavorable anhedonic state [39,40]. Arguably, individuals with a combination of the low enzyme activity Met/Met genotype have high dopamine

levels - and do not have an anhedonic state - which might be preventive for substance use.

A strength of our study is the measurement of both genetic and epigenetic variations of the *COMT* gene, which provides a more complete picture of the role of COMT in substance use. In addition, we analyzed several types of substance use that are highly prevalent in adolescence and assessed recent use to minimize recall bias, thereby gaining reliable measures for substance use. Some limitations of our study have to be noted as well. The cross-sectional nature of our study prevented us from investigating whether methylation is a consequence of substance use, or whether methylation predisposes an individual to drug seeking behavior; a question with no definitive answer in the literature thus far [41]. To this end, repeated measurements of methylation status are needed. This paper includes a multiplicity of comparisons, which increases the risk of obtaining chance findings. This is especially relevant for the analyses of the single CpG unit data. To minimize this risk we applied a Bonferroni correction. However, for the analyses including methylation data, genotypes and substance use we did not correct for multiple testing. Therefore, we were cautious with interpreting our findings and would like to emphasize that replication is warranted. It should be noted that we studied adolescents who have had a relatively short exposure to substance use. Associations may be stronger in adults who have developed a substance addiction earlier in life or have a more intense and longer history of use. Adolescents in our study may still be experimenting with drugs, and this may be motivated by different brain mechanisms than drug addiction. We were interested in methylation of the *COMT* gene in the brain, but as this is impossible to determine in a cohort study of adolescents, we used DNA from blood cells to determine methylation rates. This is probably a valid approach as identical methylation patterns for the *COMT* gene in blood and the brain were reported previously [42], which indicates that *COMT* gene methylation in blood may be used as a proxy for *COMT* gene methylation in the brain.

To conclude, we showed that methylation of the *MB-COMT* promoter was associated with non-daily smoking in adolescents. Our study further suggests that epigenetics, in combination with the *COMT* Val$^{108/158}$Met polymorphism, could be associated with cannabis use during adolescence. Maybe through altering COMT activity and gene expression, and thereby influencing the dopamine metabolism in the brain. However, this finding warrants replication in other populations, including adults and individuals who are addicted to substances. Our findings may also provide a first step in the prevention of substance use disorders. Epigenetic modifications may prove to be useful biomarkers to identify susceptibility or vulnerability for substance use, and, in time, our findings may even contribute to the development or improvement of effective behavioral or pharmacological interventions for substance use disorders. In order to obtain more insight into the mechanisms involved in substance use and abuse it may be helpful to include both genetic and epigenetic factors.

## References


1. Macleod J, Oakes R, Copello A, Crome I, Egger M, et al. (2004) Psychological and social sequelae of cannabis and other illicit drug use by young people: a systematic review of longitudinal, general population studies. Lancet 363: 1579-1588.
2. Robinson TE, Berridge KC (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev 18: 247-291.
3. Wanat MJ, Willuhn I, Clark JJ, Phillips PE (2009) Phasic dopamine release in appetitive behaviors and drug addiction. Curr Drug Abuse Rev 2: 195-213.
4. Grossman MH, Emanuel BS, Budarf ML (1992) Chromosomal mapping of the human catechol-O-methyltransferase gene to 22q11.1----q11.2. Genomics 12: 822-825.
5. Tenhunen J, Salminen M, Lundstrom K, Kiviluoto T, Savolainen R, et al. (1994) Genomic organization of the human catechol O-methyltransferase gene and its expression from two distinct promoters. Eur J Biochem 223: 1049-1059.
6. Lachman HM, Papolos DF, Saito T, Yu YM, Szumlanski CL, et al. (1996) Human catechol-O-methyltransferase pharmacogenetics: description of a functional polymorphism and its potential application to neuropsychiatric disorders. Pharmacogenetics 6: 243-250.
7. Beuten J, Payne TJ, Ma JZ, Li MD (2006) Significant association of catechol-O-methyltransferase (COMT) haplotypes with nicotine dependence in male and female smokers of two ethnic populations. Neuropsychopharmacology 31: 675-684.
8. Horowitz R, Kotler M, Shufman E, Aharoni S, Kremer I, et al. (2000) Confirmation of an excess of the high enzyme activity COMT val allele in heroin addicts in a family-based haplotype relative risk study. Am J Med Genet 96: 599-603.
9. Li T, Chen CK, Hu X, Ball D, Lin SK, et al. (2004) Association analysis of the DRD4 and COMT genes in methamphetamine abuse. Am J Med Genet B Neuropsychiatr Genet 129B: 120-124.
10. Redden DT, Shields PG, Epstein L, Wileyto EP, Zakharkin SO, et al. (2005) Catechol-O-methyltransferase functional polymorphism and nicotine dependence: an evaluation of nonreplicated results. Cancer Epidemiol Biomarkers Prev 14: 1384-1389.
11. Vandenbergh DJ, Rodriguez LA, Miller IT, Uhl GR, Lachman HM (1997) High-activity catechol-O-methyltransferase allele is more prevalent in polysubstance abusers. Am J Med Genet 74: 439-442.
12. Tammimaki AE, Mannisto PT (2010) Are genetic variants of COMT associated with addiction? Pharmacogenet Genomics 20: 717-741.
13. Abdolmaleky HM, Cheng KH, Faraone SV, Wilcox M, Glatt SJ, et al. (2006) Hypomethylation of MB-COMT promoter is a major risk factor for schizophrenia and bipolar disorder. Hum Mol Genet 15: 3132-3145.
14. Sasaki M, Kaneuchi M, Sakuragi N, Dahiya R (2003) Multiple promoters of catechol-O-methyltransferase gene are selectively inactivated by CpG hypermethylation in endometrial cancer. Cancer Res 63: 3101-3106.
15. Xu Q, Ma JZ, Payne TJ, Li MD (2010) Determination of Methylated CpG Sites in the Promoter Region of Catechol-O-Methyltransferase (COMT) and their Involvement in the Etiology of Tobacco Smoking. Front Psychiatry 1: 16.
16. Huisman M, Oldehinkel AJ, de Winter A, Minderaa RB, de Bildt A, et al. (2008) Cohort profile: the Dutch 'TRacking Adolescents' Individual Lives' Survey'; TRAILS. Int J Epidemiol 37: 1227-1235.

17. Ormel J, Oldehinkel AJ, Sijtsema J, van Oort F, Raven D, et al. (2012) The TRacking Adolescents' Individual Lives Survey (TRAILS): design, current status, and selected findings. J Am Acad Child Adolesc Psychiatry 51: 1020-1036.

18. Harakeh Z, de Sonneville L, van den Eijnden RJ, Huizink AC, Reijneveld SA, et al. (2012) The association between neurocognitive functioning and smoking in adolescence: the TRAILS study. Neuropsychology 26: 541-550.

19. Creemers HE, Harakeh Z, Dick DM, Meyers J, Vollebergh WA, et al. (2011) DRD2 and DRD4 in relation to regular alcohol and cannabis use among adolescents: does parenting modify the impact of genetic vulnerability? The TRAILS study. Drug Alcohol Depend 115: 35-42.

20. Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16: 1215.

21. Zhao AM, Cheng Y, Li XT, Li QL, Wang L, et al. (2011) Promoter hypomethylation of COMT in human placenta is not associated with the development of pre-eclampsia. Molecular Human Reproduction 17: 199-206.

22. van der Knaap LJ, Riese H, Hudziak JJ, Verbiest MM, Verhulst FC, et al. (2014) Glucocorticoid receptor gene (NR3C1) methylation following stressful events between birth and adolescence. The TRAILS study. Transl Psychiatry 4: e381.

23. Nijmeijer JS, Hartman CA, Rommelse NN, Altink ME, Buschgens CJ, et al. (2010) Perinatal risk factors interacting with catechol O-methyltransferase and the serotonin transporter gene predict ASD symptoms in children with ADHD. J Child Psychol Psychiatry 51: 1242-1250.

24. Stavrakakis N, Oldehinkel AJ, Nederhof E, Oude Voshaar RC, Verhulst FC, et al. (2013) Plasticity genes do not modify associations between physical activity and depressive symptoms. Health Psychol 32: 785-792.

25. Horvath S (2013) DNA methylation age of human tissues and cell types. Genome Biol 14: R115.

26. Becker JB, Hu M (2008) Sex differences in drug abuse. Front Neuroendocrinol 29: 36-47.

27. Johansson A, Enroth S, Gyllensten U (2013) Continuous Aging of the Human DNA Methylome Throughout the Human Lifespan. PLoS One 8: e67378.

28. Hillemacher T, Frieling H, Hartl T, Wilhelm J, Kornhuber J, et al. (2009) Promoter specific methylation of the dopamine transporter gene is altered in alcohol dependence and associated with craving. J Psychiatr Res 43: 388-392.

29. Philibert RA, Gunter TD, Beach SR, Brody GH, Madan A (2008) MAOA methylation is associated with nicotine and alcohol dependence in women. Am J Med Genet B Neuropsychiatr Genet 147B: 565-570.

30. Brook JS, Zhang CS, Brook DW (2011) Developmental Trajectories of Marijuana Use From Adolescence to Adulthood Personal Predictors. Archives of Pediatrics & Adolescent Medicine 165: 55-60.

31. Marschall-Levesque S, Castellanos-Ryan N, Vitaro F, Seguin JR (2014) Moderators of the association between peer and target adolescent substance use. Addictive Behaviors 39: 48-70.

32. Teichman M, Barnea Z, Ravav G (1989) Personality and Substance Use among Adolescents - a Longitudinal-Study. British Journal of Addiction 84: 181-190.

33. Golimbet VE, Alfimova MV, Gritsenko IK, Ebstein RP (2007) Relationship between dopamine system genes and extraversion and novelty seeking. Neurosci Behav Physiol 37: 601-606.

34. Demetrovics Z, Varga G, Szekely A, Vereczkei A, Csorba J, et al. (2010) Association between Novelty Seeking of opiate-dependent patients and the catechol-O-methyltransferase Val(158)Met polymorphism. Compr Psychiatry 51: 510-515.

35. Hosak L, Libiger J, Cizek J, Beranek M, Cermakova E (2006) The COMT Val158Met polymorphism is associated with novelty seeking in Czech methamphetamine abusers: preliminary results. Neuro Endocrinol Lett 27: 799-802.

36. Lang UE, Bajbouj M, Sander T, Gallinat J (2007) Gender-dependent association of the functional catechol-O-methyltransferase Val158Met genotype with sensation seeking personality trait. Neuropsychopharmacology 32: 1950-1955.

37. Strobel A, Lesch KP, Jatzke S, Paetzold F, Brocke B (2003) Further evidence for a modulation of Novelty Seeking by DRD4 exon III, 5-HTTLPR, and COMT val/met variants. Mol Psychiatry 8: 371-372.

38. Wise RA (1978) Catecholamine theories of reward: a critical review. Brain Res 152: 215-247.

39. Markou A, Koob GF (1991) Postcocaine anhedonia. An animal model of cocaine withdrawal. Neuropsychopharmacology 4: 17-26.

40. Volkow ND, Wang GJ, Fowler JS, Tomasi D, Telang F, et al. (2010) Addiction: decreased reward sensitivity and increased expectation sensitivity conspire to overwhelm the brain's control circuit. Bioessays 32: 748-755.

41. Nielsen DA, Utrankar A, Reyes JA, Simons DD, Kosten TR (2012) Epigenetics of drug abuse: predisposition or response. Pharmacogenomics 13: 1149-1160.

42. Murphy BC, O'Reilly RL, Singh SM (2005) Site-specific cytosine methylation in S-COMT promoter in 31 brain regions with implications for studies involving schizophrenia. Am J Med Genet B Neuropsychiatr Genet 133B: 37-42.

## Supplementary information

For the MB-COMT genomic region, we used a primer set that was previously reported by Xu et al., (2010). While Xu et al. present the exact positions of CpG sites relative to the transcription start site, in the current study we present CpG units that contained one or multiple CpG sites. The overlap between the CpG sites as presented by Xu et al. (2010) and the CpG units in the current study is shown in Table S1.



**MB-COMT**

5'-tggg|at|acc|agctctggg|ag|acc|ac|aggtgc|agtc|agc|ac|agc|agg|accttt|ag|ac|a|aggc|accc|agcccc|agtttcccc|acctggg|a|agggggct|acttgtggct|ag|a|agc|AGCC$^{243}$**CG**G$^{1}$|actcctg|agc|a|ag|act|ag|acc|a|ag|AGGC$^{211}$**CG**GT$^{2}$|atgtgg|ac|ACCCC$^{195}$**CG**$^{193}$**CG**TGGGC$^{3}$|accccc|A$^{179}$**CG**GGG$^{4}$|ac|accctggcc|AC$^{161}$**CG**C$^{-158}$**CG**$^{156}$**CG**$^{154}$**CG**G$^{5}$|ac|accctc|A$^{142}$**CG**$^{6}$|agg|ac|ACCC$^{131}$**CG**GC$^{127}$**CG**$^{125}$**CG**$^{123}$**CG**G$^{7}$|ac|acct|AC$^{112}$**CG**$^{-110}$**CG**GGG$^{8}$|A$^{104}$**CG**CCC$^{99}$**CG**$^{9}$|acccc|atcct|ACCTGCTG$^{79}$**CG**CCC$^{74}$**CG**$^{72}$**CG**C$^{69}$**CG**$^{67}$**CG**CCC$^{62}$**CG**C$^{10}$|ACCC$^{-55}$**CG**CC$^{51}$**CG**CC$^{11}$|A$^{46}$**CG**GCCTG$^{39}$**CG**TC$^{35}$**CG**CC$^{12}$|AC$^{29}$**CG**G$^{13}$|a|AG$^{23}$**CG**CCCTCCT$^{14}$|a|ATCCC$^{8}$**CG**C$^{15}$|AG$^{-3}$**CG**C$^{16}$|AC$^{+4}$**CG**CC$^{17}$|ATTGC**CG**CC$^{18}$|AT**CG**T**CG**TGGGGCTTCTGGGGC$^{19}$|agct|agggctgcc -3'

**S-COMT**

5'-gtgggtgctgc|agg|agg|agc|ac|ag|agc|ACTGG**CG**CCCCTCCCCTCC**CG**CCCTGC$^{1}$|ag|ATGC**CG**G$^{2}$|AGGCCC**CG**CCTCTGCTGTTGGC$^{3}$|agctgtgttgctgggcctggtgctgctggtggtgctgctgctgcttctg|aggc|actggggctggggcctgtgcctt|AT**CG**GCTGG$^{4}$|a|A**CG**$^{5}$|agttc|atcctgc|agccc|atcc|ac|a|acctgctc|atgggtg|ac|acc|a|agg|agc|AG**CG**C$^{6}$|atcctg|a|acc|A**CG**TGCTGC$^{7}$|agc|ATG**CG**G$^{8}$|AGCC**CG**GG$^{9}$|a|A**CG**C$^{10}$|ac|ag|AG**CG**TGCTGG$^{11}$|aggcc|attg|ac|acct|ACTG**CG**$^{12}$|agc|ag|a|agg|agtgggcc|atg|a|A**CG**TGGG**CG**$^{13}$|ac|a|ag|a|a|AGGTGGGGTC**CG**GGCC$^{14}$|agc|aggtgctc|agctctggg|ac|aggg|accc|agg|acc|aggc|a -3'

**Figure S1.** The sequence and position of individual CpG sites and CpG units is shown below for both MB-COMT (upper panel) and S-COMT (lower panel) DNA fragments. The vertical lines represent the position of splice sites on the complementary RNA strand (not shown), and any fragment containing one or more CpG sites (bold letters) is considered a CpG unit and are represented by uppercase letters and numbered at the right end side. For MB-COMT, the individual CpG sites are labeled by their relative position in relation to the transcription start site (+1, the underlined C in CpG unit 16) according to . Methylation rates for grey CpG units could not be obtained or used for analyses (see method section for further details).

**Table S1:** Overlap between CpG units in the current study and CpG sites reported by Xu et al., 2010.

| CpG units presented in our study | CpG site positions relative to the transcription start site as presented by Xu et al. | | | |
|---|---|---|---|---|
| MB-COMT CpGU2 | -211 | | | |
| MB-COMT CpGU3 | -195[a] | -193[b] | | |
| MB-COMT CpGU5 | -161 | -158 | -156[a] | -154[a] |
| MB-COMT CpGU7 | -131[a] | -127[a] | -125 | -123[a] |
| MB-COMT CpGU8 | -112[a] | -110 | | |
| MB-COMT CpGU9 | -104[a] | -99[a] | | |
| MB-COMT CpGU11 | -55 | -51 | | |
| MB-COMT CpGU12 | -46[a] | -39[b] | -35[a] | |
| MB-COMT CpGU14 | -23 | | | |
| MB-COMT CpGU15 | -8[a] | | | |
| MB-COMT CpGU19 | +19[a] | +22[a] | | |

Note: most units contain more than one CpG.

[a] No information on methylation in Xu et al., 2010

[b] Difference found between smokers and non smokers in Xu et al., 2010.

Units printed in bold represent units that were significantly related to substance use (non-daily smoking) in the current analyses.

**Table S2.** CpG Unit descriptive statistics: methylation rates of the individual CpG units.

| | N | Minimum | Maximum | Mean | Std. Deviation |
|---|---|---|---|---|---|
| MB-COMT CpGU2 | 458 | 0% | 3% | 0.1% | 0.4% |
| MB-COMT CpGU3 | 445 | 1% | 19% | 8.9% | 3.4% |
| MB-COMT CpGU5 | 457 | 0% | 6% | 0.8% | 0.8% |
| MB-COMT CpGU7 | 456 | 2% | 10% | 5.2% | 1.5% |
| MB-COMT CpGU8 | 436 | 2% | 14% | 5.4% | 1.4% |
| MB-COMT CpGU9 | 458 | 0% | 6% | 1.9% | 0.8% |
| MB-COMT CpGU11 | 458 | 0% | 9% | 3.3% | 1.7% |
| MB-COMT CpGU12 | 456 | 0% | 6% | 2.1% | 0.9% |
| MB-COMT CpGU14 | 456 | 0% | 8% | 0.9% | 1.0% |
| MB-COMT CpGU15 | 458 | 0% | 8% | 1.0% | 0.9% |
| MB-COMT CpGU19 | 455 | 2% | 9% | 4.4% | 1.1% |
| S-COMT CpGU8 | 362 | 70% | 100% | 93.1% | 4.3% |
| S-COMT CpGU11 | 447 | 87% | 100% | 97.5% | 2.0% |
| S-COMT CpGU12 | 366 | 65% | 98% | 86.8% | 4.8% |
| S-COMT CpGU13 | 456 | 88% | 100% | 95.7% | 2.2% |
| S-COMT CpGU14 | 437 | 89% | 100% | 97.8% | 1.8% |

## References

1. Xu Q, Ma JZ, Payne TJ, Li MD (2010) Determination of Methylated CpG Sites in the Promoter Region of Catechol-O-Methyltransferase (COMT) and their Involvement in the Etiology of Tobacco Smoking. Front Psychiatry 1:16.

# chapter 8

Discussion

The aim of this thesis was to investigate the connections between physiological and psychological stress reactivity and externalizing and internalizing problems in adolescence. To this end, we focused on the role of new biological mechanisms, namely, autoantibodies against neuropeptides and gene methylation, and their possible role in stress reactivity and externalizing and internalizing problems. Another focus was to investigate the relationship between stressful life events and stress reactivity and cannabis use in adolescence. In this chapter the main results of this thesis as well as methodological challenges and implications of the findings are discussed.

## About neuropeptide autoantibodies, HPA axis reactivity and problem behavior



Chapter 2 describes that higher levels of free ACTH-reactive autoantibodies (IgG) impair HPA axis reactivity in boys. In chapter 4, we also show that higher levels of total ghrelin-reactive IgG were related to a lower HPA axis reactivity in girls, albeit weakly. There are no previous studies on the relationship between neuropeptide autoantibodies and HPA axis reactivity, hence a comparison with existing literature is not possible. On the one hand, a relationship between ACTH autoantibodies and HPA axis reactivity is biologically plausible. ACTH is an important component of the HPA axis, as it stimulates the release of cortisol from the adrenal glands. When ACTH is bound by antibodies (i.e., in an immune complex) it is likely that this will have a direct effect on subsequent cortisol release. On the other hand, the way ghrelin IgG could influence HPA axis reactivity is less obvious. While one study in mice has shown that ghrelin can stimulate HPA axis reactivity at the anterior pituitary [1], we do not know of any human studies. Considering the probably weak influence of ghrelin on HPA axis reactivity and the fact that multiple comparisons in our study in chapter 4 might have led to chance findings, it is unlikely that ghrelin autoantibodies have a strong effect on HPA axis regulation in humans. The influence of ACTH autoantibodies on the regulation of HPA axis reactivity is a more promising finding, although it is important to note that the effects we found were not strong. The HPA axis is regulated by multiple factors, including genetics [2], epigenetics [3], cytokines [4], prenatal and early life stress [5,6]. Taking this into consideration, autoantibodies against ACTH are just one of the many factors regulating HPA axis reactivity and therefore probably play a modest role.

We also studied autoantibodies against neuropeptides in relation to problem behavior. ACTH autoantibodies were associated with antisocial behavior problems in boys (chapter 2), and with internalizing behavior problems in girls (chapter 3). These findings are in line with previous studies that have shown that ACTH autoantibodies were associated with conduct disorders in males and maturity fear in female patients with eating disorder [7,8]. Our results are a valuable extension of the existing literature, because previous studies were done in specific populations,

while we made use of a large sample from the general population, which makes our results more generalizable. The likely mechanism by which ACTH IgG could affect externalizing and internalizing problems is via inducing changes in the HPA axis. The HPA axis has been implicated in both the development of externalizing and internalizing problems [9,10], hence, by affecting the cortisol response during stress, ACTH IgG could also affect problem behavior. However, the biological mechanisms behind these associations have to be further studied before such a conclusion can be drawn. While previous studies also found associations between α-MSH and anxiety in rats and in patients with an autoimmune disorder [11,12], we could not confirm these findings in our study (chapter 3). Differences might have resulted from the use of specific population samples or different internalizing symptoms in those studies. As for ghrelin autoantibodies, we found a weak relationship with anxiety symptoms in girls (chapter 4). Some animal experiments have shown that ghrelin has anti-depressant effects and can restrict anxiety symptoms [1,13], while others have shown the opposite effect [14-16]. So far, there is only weak evidence from human studies that ghrelin might protect against anxiety or depression [17]. Another study found higher levels of ghrelin in depressed patients, which normalized after treatment [18]. In our study, there was no association between ghrelin autoantibodies and depressive symptoms, which are highly correlated with anxiety symptoms. In addition, the possible biological mechanism by which ghrelin autoantibodies could be only connected to anxiety symptoms is not clear. Due to the explorative nature of this study and a lack of a biological explanation we have to be careful with interpreting these results, as they could be chance findings.



In all three of our studies on autoantibodies against neuropeptides, sex acted as a moderator. Interestingly, girls had higher levels of all autoantibodies that we measured. In general, women are known to have higher antibody levels than men [19-21], probably due to differences in immune system function and hormones such as estrogens, which can affect antibody production [22]. Comparable results have been found in rats, where ACTH and α-MSH autoantibody levels were higher in females than in males, and differences in gut bacteria composition were proposed as underlying reason [23]. Considering the molecular mimicry hypothesis as a basis for the formation of neuropeptide autoantibodies, it would be interesting to further study whether differences in the microbiome in boys versus girls are responsible for the differences in levels of neuropeptide autoantibodies. It is well known that there are differences in HPA axis reactivity between boys and girls [24]. In addition, girls generally have higher levels of internalizing problems than boys, while boys more often develop externalizing problems [25,26]. Moreover, sex has been found to moderate the relationships between HPA axis functioning and externalizing or internalizing problems [27,28]. Hence, it is not very surprising to find sex as moderating factor in the relationship between autoantibodies and HPA axis reactivity and problem behavior, but the underlying reasons remain to be further elucidated.

## Neuropeptide autoantibodies – general considerations

This thesis presents the first studies of neuropeptide autoantibodies in relation to stress reactivity and problem behavior in the general population. While our findings indicate involvement of autoantibodies in both physiological and psychological mechanisms, this field of research is still in its infancy. I would like to add a few considerations regarding our and future research on neuropeptide autoantibodies.

First, we found promising relationships between ACTH IgG levels and HPA axis reactivity as well as problem behavior. However, to interpret these findings correctly it is crucial to determine the working mechanism of ACTH IgG, and this should be of primary concern in future studies. Two working mechanism have been suggested for neuropeptide autoantibodies, neutralization and transportation. Our results, on first sight, support the theory that free ACTH IgG neutralizes ACTH by free ACTH IgG and thereby impair the cortisol response in a situation of acute stress. However, one could argue that binding capacities of free ACTH IgG are low and therefore they fail to ´protect´ ACTH from degradation, resulting in a blunted cortisol response. The latter mechanism is supported by findings for ghrelin autoantibodies in rats, which `protected` ghrelin from degradation and thereby enhanced its orexigenic effect [29]. It is impossible to draw conclusions about a working mechanism of ACTH autoantibodies merely from association studies, thus, experiments are needed. Whether neutralization or transportation applies might be determined by the affinity of the autoantibodies [12,30]. We therefore attempted to further elucidate the working mechanism of ACTH IgG by measuring binding affinities, albeit without success (chapter 2). To elucidate the working mechanism of ACTH autoantibodies, functional mechanistic studies in animals could be suitable [29]. However, in-vitro possibilities should also be further pursued, e.g., using cell culture based assays of receptor activation by antibodies [23]. One should keep in mind that different mechanism might apply for different peptides and different classes (e.g., IgG versus IgM or IgA) and sub-classes of antibodies (e.g., IgG1 versus IgG2), due to their different binding properties or stages of affinity maturation [31].

Second, we measured ACTH autoantibody levels both in physiological condition (´free´ IgG), and in dissociating condition (´total´ IgG) [30]. Free and total levels of autoantibodies were highly correlated, but still showed differential associations with antisocial behavior, internalizing behavior or HPA axis reactivity (chapter 2 and 3). In particular, the relationship with antisocial or internalizing behavior depended on the interaction between free and total ACTH IgG. The reason for this interaction is to be further elucidated. In addition, the level of free autoantibodies depends on the level of antigen. It is therefore important to note that antigen (in this case ACTH) levels could have been an important confounder in the relationship between ACTH autoantibodies and HPA axis reactivity. Thus, one limitation of our studies in chapters 2 – 4 is that we did not measure the levels of ACTH, α-MSH and ghrelin in all subjects.

Third, even though autoantibodies are usually associated with autoimmune disease, we harbour some autoantibodies from birth onwards [32], and there is a

possibility that they might serve a physiological function (e.g., regulation of HPA axis reactivity), such as shown for other ´natural autoantibodies´ [33,34]. Yet, the reasons for the formation of such autoantibodies are not fully understood. By showing that ACTH, α-MSH and ghrelin autoantibodies correlate with influenza A antibodies (chapter 3 and 4), our study provides additional evidence for the molecular mimicry hypothesis, according to which neuropeptide autoantibodies are formed due to sequence similarities with pathogenic peptides [35]. Interestingly, infections and autoantibody formation have long been known to play a role in neuropsychiatric diseases, e.g., Sydenham chorea, an autoimmune disease linked to infection with Group A Streptococci [36,37]. This finding laid the base for the concept of molecular mimicry as source of neuropsychiatric symptoms which involve the formation of autoantibodies against brain antigens. Subsequently, the hypothesis of a pediatric autoimmune disorder called ´PANDAS syndrome´ (Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections) developed. Symptoms include for example sudden onset of tics and obsessive compulsive behavior [38-40]. It has not been conclusively proven, but a connection between autoantibodies against Group A Streptococci that cross-react with the basal ganglia in the brain could be a cause of PANDAS [39,41]. Moreover, antibodies against brain proteins or peptides were found in children with autism spectrum disorder, but not all targets have been identified [42,43]. It could thus be that neuropeptide autoantibodies are – in part - formed due to infections and then influence physiological functions, increasing the risk of developing psychiatric problems.



Finally, our findings are interesting in light of the microbiota-gut-brain axis, which recently has received lots of attention [44]. The microbes in our gut have an influence on our immune system, psychological and physiological mechanisms, via neuropeptides, cytokines, and endocrine hormones [45], and changes in the gut microbiome have been linked to autism and depression [46,47]. Even HPA axis development is influenced by our gut microbiome, yet the mechanisms have not been fully elucidated [48]. Maybe our study provides a hint in the puzzle as to how microbes might be able to affect HPA axis reactivity.

We have to be careful with the interpretation of our studies, as they are the first to show autoantibodies against neuropeptides in relation to behavioural and emotional problems in adolescence. Nevertheless, our findings indicate that investigating neuropeptide autoantibodies could provide interesting information as to how infections could be linked to stress-related mental health problems.

## About stressful life events, stress reactivity and substance use

Stress is related to problem behavior in different ways, e.g., via stressful life events (SLE), but also via Hypothalamus-pituitary-adrenal (HPA) axis reactivity [49-52]. While studies have shown that both SLE and HPA axis reactivity are related to both externalizing and internalizing problems in adolescents [9,53,54], there is still controversy about the directionality of the relationship as well as the underlying biological mechanisms

[10,55,56]. Importantly, in chapter 4 we show that there appears to be a bidirectional relationship between SLE and cannabis use. This study is the first to show reciprocal associations between SLE and cannabis use in a large general population sample of adolescents. Our result that SLE predict later cannabis use confirms results of previous studies [57-59]. Vice versa, much is also known about the negative consequences of regular cannabis use such as problems with family, friends, higher crime rates and lower education levels due to cannabis use [60]. The relationships we found were attenuated by adding adolescent´s externalizing problems to the model, which is not very surprising, as cannabis use and externalizing problems are highly correlated (chapter 5). However, this could also be due to a mediating role of externalizing problems between SLE and cannabis use [54]. We have to keep in mind that in the study of SLE and cannabis use, many factors play a role as moderators and mediators, and it is difficult to discern relationships between specific predictors and outcomes. For example, adolescent smoking has been suggested to be a mediator between externalizing problems and cannabis use [61]. While studying the multifactorial mechanism of cannabis use and SLE in adolescents, there is a risk to over-adjust in statistical models [62]. In addition, it would be important to also study factors that can prevent SLE and cannabis use, and factors that influence whether someone continues or stops using cannabis, as knowing those factors could help to develop prevention programs [63]. One of those factors could be stress reactivity, which was studied in chapter 6.



The results of our study on HPA axis reactivity and future problematic cannabis use did not match our hypothesis, neither the results of other studies (chapter 6). Even though previous findings from TRAILS show that a blunted HPA axis reactivity was associated with earlier initiation of cannabis use as well as with current cannabis use [64,65], we did not find any relationship between HPA axis reactivity and problematic cannabis use in our prospective study. Instead, we found that in current cannabis users there was an association between high perceived arousal in anticipation of a stressor and later problematic cannabis use (chapter 6). It might be that adolescents with high perceived stress levels are more likely to use cannabis to reduce tension and negative affect and therefore have a higher risk of future cannabis use problems. Although this is an interesting topic for future studies, we have to keep in mind that it could be a chance finding. The lack of association between HPA axis reactivity and problematic cannabis use was surprising, yet, it could be explained by the following considerations: There was a six year gap between HPA axis reactivity measurement and the assessment of problematic cannabis use. This is a long time period, especially considering the changes the HPA axis undergoes during puberty [66-68]. For example, a recent study found that the relationship between HPA axis reactivity and the risk of developing major depressive disorder (MDD) was moderated by pubertal stage [69]. And, one study in a sub-sample of TRAILS participants showed that HPA axis reactivity was increased at age 19 compared to age 16 (Laceulle et.al, submitted). Repeated measurements of both HPA axis reactivity and behavior and emotional problems in

adolescence would help to gain a better understanding of the possibly reciprocal associations and underlying mechanisms. Another important consideration is that different mechanisms are likely to play a role in the transition from no cannabis use to regular cannabis use (i.e. initiation) versus the transition from regular cannabis use to problematic cannabis use [70,71]. Here, we investigated the latter, while previous studies on the relationship between HPA axis reactivity and cannabis use mostly focused on regular versus no use or initiation [64,65]. Maybe HPA axis reactivity plays a role in this particular transition but not in the transition to problematic cannabis use. It is also likely that the associations we tested are mediated and moderated by factors that affect both externalizing behavior and stress reactivity, such as parental substance use [72], SLE [53], temperament [73] and genetics [74,75]. Moreover, gene-environment interactions involving HPA axis genes and SLE might be involved in the development of mood disorders [76], hence, they might also play a role in the development of problematic substance use. Finally, gene methylation could play a role in substance use, therefore we tested this assumption in chapter 7.



## About gene methylation and substance use

Studies on the role of the COMT single nucleotide polymorphism Val$^{108/158}$Met in substance use and addiction have led to conflicting results [77]. To shed light on this issue, we investigated whether methylation (the addition of a methyl group to DNA) of the COMT gene in combination with the Val$^{108/158}$Met polymorphism was related to substance use. We also investigated whether mean methylation levels or methylation at different CpG sites of the COMT gene were related to substance use. So far, one study has linked alcohol use to increased *MB-COMT* promoter methylation [78]. Another study has shown that daily smoking was related to increased methylation at two specific CpG sites in the *MB-COMT* promoter [79]. Our findings add that overall methylation of the *MB-COMT* promoter is associated with non-weekly smoking (chapter 7). We also found an association between methylation at one of the two CpG sites studied previously by Xu. et al. and non-daily smoking [79]. We did not find an association between COMT gene methylation and alcohol or cannabis use. The differences between ours and previous studies could be due to the fact that our study included younger subjects with a shorter history of smoking. One remaining question concerns the directionality of this relationship. Smoking has been identified as environmental factor that can affect methylation status [80-82]. However, from our cross-sectional study we cannot conclude whether methylation was a consequence of smoking or vice versa. To address this question, longitudinal studies with repeated measurements of both methylation levels and substance use are needed. Preferably, this could be done in birth cohorts, to be able to assess the dynamics of methylation prenatally and throughout adolescence, taking into account changes in methylation at different time points. Even maternal smoking has been shown to affect methylation status of the embryo [80], though not specifically for *MB-COMT* promoter methylation. Nevertheless, the cross-generational influence of epigenetic modifications is widely

recognized [83]. In addition, substance use also often occurs within families [84]. Epigenetics might thus be a link between parental and offspring substance use, but this hypothesis needs to be further studied. It has to be added that the association between COMT methylation and non-weekly smoking in our study might be a chance finding. A limitation of this study was that we did not measure the expression of COMT, thus we cannot confirm that COMT levels were actually lower in adolescents with high methylation levels. Neither do we know whether methylation of COMT was indeed related to higher dopamine levels in our study, hence, the functional relevance of COMT methylation needs to be further addressed. However, previous studies have shown that methylation was linked to decreased COMT expression and suggest higher dopamine levels as result [78,85].



We also showed that adolescents with the Met/Met genotype and high levels of *MB-COMT* promoter methylation had a lower risk of cannabis use than adolescents with the Val/Val or Val/Met genotypes. This could be due to higher dopamine levels resulting from an inactive form of COMT and low levels of expression. While there is controversy about whether the $Val^{108/158}$Met polymorphism presents a risk factor for substance use, these results suggest that it could be important to not only study polymorphisms in relation to substance use, but also gene methylation, as it might moderate the relationship between polymorphisms and substance use. Especially in adolescence, which presents a window for both substance use initiation and gene methylation [86], this might be a valuable tool for predicting adolescent substance use and eventually, it might even become a target for intervention. Gene methylation increases not only with age [87,88], but it has also been linked to exposure to SLE, particularly during adolescence [86]. Furthermore, gene methylation has been linked to HPA axis functioning [3]. Methylation therefore might be a mediating factor between SLE, HPA axis reactivity and substance use. This could be an interesting topic for future studies.

## Methodological considerations

The *study design* we used to investigate autoantibodies and methylation were of cross-sectional nature (chapters 2-4 and 7). One of the major limitations of cross-sectional studies is that we cannot draw conclusions as to whether the biological factors preceded problem behavior or vice versa. To this end, longitudinal studies are needed with multiple measurements of both the biological factors (autoantibodies and methylation) and the outcomes. Another option to further elucidate possible pathways would be to use mediation models to study, for example, whether autoantibodies mediate associations between influenza infection and anxiety or depression. Or, whether COMT methylation mediates the relationship between SLE and substance use.

Our *study sample* consisted of 2230 adolescents from the general population, representing all ranges of substance use risk and levels of SLE and stress reactivity. One of the factors that could have contributed to a lack of associations that we

initially expected could have been that previous studies used clinical samples, while our study focused on the general population with a much lower risk profile. For example, ghrelin and α-MSH antibodies had been related to internalizing problems in patients with eating disorders and an immune disorder, who are generally more likely to have other psychological problems such as depression or anxiety disorders [7,11]. Furthermore, our study sample was on average younger than the previously studied samples, which might have contributed to the differences in results, as IgG levels increase with age [19]. Having said this, our study sample also has advantages. We tested associations in a large and representative sample of adolescents, making findings more generalizable than studies in clinical samples. In addition, our large sample size made it possible to detect small effects and to test the influence of many different covariates and interactions in our analyses.



*Measuring stress* is a difficult task and has been a topic of discussion due to the diverse characteristics of stress and different definitions of stress [89]. In this study, we focused on stressful life events and the physical and psychological stress response. While we only selected negative SLE, they can be perceived differently between individuals, thus we do not know the true impact of an SLE on an individual adolescent. This might have affected our analyses, as a more negative perception of an SLE might contribute more to the risk of cannabis use. In addition, it might be that adolescents experienced other negative events that we did not measure, or that recall bias occurred while measuring SLE retrospectively. These two scenarios would probably have led to an underestimation of the effects of SLE on cannabis use. The Groningen social stress test (GSST), which was used in three of the studies presented in this thesis, is an efficient way to elicit a cortisol response in adolescents (chapters 2, 4 and 5) [90]. We tested possible confounders, such as smoking, use of oral contraceptives and medication, but we cannot exclude that other confounding factors remained that were not adjusted for, e.g., genetic factors [75,76]. Finally, as mentioned before, HPA axis reactivity changes during puberty due to sexual maturation [66-68], therefore measuring during adolescence might provide a rather unstable snapshot of HPA reactivity. It would be preferable to have repeated measurements of HPA axis reactivity available, to study the changes of HPA axis reactivity and cannabis use in relation to each other.

*Substance use* is often initiated during adolescence, and early initiation increases the risk of future substance use problems. This makes it a very interesting period to study the development of problematic substance use, which could lead to addiction. However, only few adolescents who use substances, e.g., cannabis, during their adolescent years, become addicted [91]. It is important to distinguish factors that predict initiation of substance from factors that affect the development of addiction. Particularly the transition from regular to problematic substance use should be focus of future studies, as problematic use is associated with severe consequences. Another problem lies in the definitions of different types of substance use, e.g., ´regular´ versus ´problematic´ substance use. Here, merely taking into consideration the number of

times that a substance is used per month or year, is probably not sufficient [92]. It comes down to the problems caused by taking a substance, making screening tests such as the CUPIT very important tools in identifying whether someone is at risk of developing a cannabis dependence or not. One limitation is that the CUPIT has so far not been validated for the Netherlands. Other similar tests, such as the Cannabis Use Disorders Identification Test (CUDIT) [93], are available. One should be aware that different tests probably identify different groups of cannabis users [94], therefore a more thorough comparison of the potential of such tests to predict cannabis use disorders among adolescents and young adults would be useful.

*Missing data* is a common problem in longitudinal cohort studies. However, many epidemiological studies fail to adequately assess and handle missing data [95]. This can lead to biased results, in the case data are not ´missing completely at random´ (MCAR), which is hardly ever the case. When data are ´missing at random´ (MAR), then methods such as multiple imputation or full information maximum likelihood are adequate to deal with such a problem and give less biased estimates [96,97], both leading to similar results in longitudinal studies [98,99]. Therefore, we used such methods wherever we had to deal with lots of missing data. The main reason for missing data in our studies was non-response to questionnaires or interviews, and adolescents who did not respond differed in some study characteristic from the individuals who did respond. In part, these differences could be explained by other variables in our analyses, therefore we believe that by using multiple imputation (MI) or full information maximum likelihood (FIML) we reduced bias due to missing data. However, the addition of auxiliary variables can further improve multiple imputation of variables, which can lead to more precise estimates [100]. While we did our best to deal with missing data in our studies, the possibility remains that bias due to missing data was not fully resolved.



## Implications and future research

Research on autoantibodies against neuropeptides and their influence on physiological systems and behavior is still in its beginnings. Mostly animal studies have been carried out, and those are not directly comparable to human studies. The studies presented in this thesis are the first that relate autoantibodies against ACTH, α-MSH or ghrelin to HPA axis reactivity, externalizing or internalizing problems in a sample of adolescents from the general population. As our findings are very preliminary, no immediate implications can be defined. However, results from this thesis indicate that it could be worthwhile to further investigate the role of such antibodies in problem behavior and in the regulation of HPA axis reactivity. First of all, more studies should be carried out to confirm the associations reported in this thesis. These should include not only general population samples but also clinical samples, e.g., adolescents and adults with conduct disorder, major depression or anxiety disorders. Knowing such associations exist could help in determining someone´s risk of developing externalizing or internalizing disorders by looking at autoantibody levels in addition

to other biomarkers, interviews and questionnaires [101]. The next step would be to further investigate the working mechanism of such autoantibodies. Here, studying the effect of autoantibodies on antigen receptor binding and animal or in-vitro studies on cell culture level could be helpful [23]. Only once the working mechanism of autoantibodies against neuropeptides is established, one could start thinking about future clinical implications, e.g., therapies, or ways to prevent or stimulate production of such autoantibodies. To this end, it might be relevant to further study the aspect of production of autoantibodies according to the molecular mimicry concept and try to pinpoint pathogenic proteins that are responsible for the formation of these autoantibodies. This is best done using animal models [23,102], or longitudinal studies in humans with repeated measures of infection and autoantibodies. Another interesting lead to pursue therefore is the connection between stress, antibody levels and problem behavior, as a study in rats has shown that levels of autoantibodies against α-MSH increased after exposure to stress [12,103]. Hence, neuropeptide autoantibodies might actually be mediators in the relationship between SLE and problem behavior or between SLE and HPA axis reactivity.



Our studies on stress reactivity and cannabis use suggest that HPA axis reactivity does not predict whether someone will develop problematic cannabis use. Thus, the relevance of HPA axis reactivity in predicting problematic substance use or addiction is questionable. The question of why HPA axis reactivity is dysregulated in substance users remains open, but it might be that it is rather substance use that modifies HPA axis reactivity than the other way around. This needs to be further investigated using repeated measures of HPA axis reactivity. At the same time, perceived stress was related to later problematic cannabis use in adolescents in our study. The perceived severity of a stressor as well as coping skills and support of the environment are important determinants for whether the stressor has a high or low impact on someone, and hence also whether someone is likely to initiate substance use following a stressor. This should be taken into account whenever studying SLE or stress reactivity. Adolescence is a period during which coping skills develop, friendships or romantic relationships become more important pillars of support, making it more difficult to measure the real impact of SLE. Hence, an interesting topic of study would also be the development of coping skills and how they mediate or moderate the relationship between SLE or perceived arousal and development of substance use [104]. Preventing high perceived stress and teaching coping strategies could maybe reduce the burden of problematic cannabis use among young adults. Furthermore, not only the transitions into cannabis use, but also the transitions away from cannabis use, i.e., desistance from use, deserves attention [63]. Knowing which factors influence whether adolescents stop using substances could help develop intervention programs or guidelines for prevention. Future studies should dig deeper into the question of why some adolescents develop problematic substance use while others do not. To this end, a combination of genetic, epigenetic and environmental factors including parental factors, SLE and temperament, should be considered.

## References


1. Spencer SJ, Xu L, Clarke MA, Lemus M, Reichenbach A, et al. (2012) Ghrelin regulates the hypothalamic-pituitary-adrenal axis and restricts anxiety after acute stress. Biol Psychiatry 72: 457-465.
2. Wust S, Federenko I, Hellhammer DH, Kirschbaum C (2000) Genetic factors, perceived chronic stress, and the free cortisol response to awakening. Psychoneuroendocrinology 25: 707-720.
3. van der Knaap LJ, Oldehinkel AJ, Verhulst FC, van Oort FV, Riese H (2015) Glucocorticoid receptor gene methylation and HPA-axis regulation in adolescents. The TRAILS study. Psychoneuroendocrinology 58: 46-50.
4. Turnbull AV, Rivier CL (1999) Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. Physiol Rev 79: 1-71.
5. Entringer S, Kumsta R, Hellhammer DH, Wadhwa PD, Wust S (2009) Prenatal exposure to maternal psychosocial stress and HPA axis regulation in young adults. Horm Behav 55: 292-298.
6. Gunnar MR, Quevedo KM (2008) Early care experiences and HPA axis regulation in children: a mechanism for later trauma vulnerability. Prog Brain Res 167: 137-149.
7. Fetissov SO, Harro J, Jaanisk M, Jarv A, Podar I, et al. (2005) Autoantibodies against neuropeptides are associated with psychological traits in eating disorders. Proc Natl Acad Sci U S A 102: 14865-14870.
8. Fetissov SO, Hallman J, Nilsson I, Lefvert AK, Oreland L, et al. (2006) Aggressive behavior linked to corticotropin-reactive autoantibodies. Biol Psychiatry 60: 799-802.
9. Hastings PD, Shirtcliff EA, Klimes-Dougan B, Allison AL, Derose L, et al. (2011) Allostasis and the development of internalizing and externalizing problems: changing relations with physiological systems across adolescence. Dev Psychopathol 23: 1149-1165.
10. Ruttle PL, Shirtcliff EA, Serbin LA, Fisher DB, Stack DM, et al. (2011) Disentangling psychobiological mechanisms underlying internalizing and externalizing behaviors in youth: longitudinal and concurrent associations with cortisol. Horm Behav 59: 123-132.
11. Karaiskos D, Mavragani CP, Sinno MH, Dechelotte P, Zintzaras E, et al. (2010) Psychopathological and personality features in primary Sjogren's syndrome--associations with autoantibodies to neuropeptides. Rheumatology (Oxford) 49: 1762-1769.
12. Sinno MH, Do Rego JC, Coeffier M, Bole-Feysot C, Ducrotte P, et al. (2009) Regulation of feeding and anxiety by alpha-MSH reactive autoantibodies. Psychoneuroendocrinology 34: 140-149.
13. Lutter M, Sakata I, Osborne-Lawrence S, Rovinsky SA, Anderson JG, et al. (2008) The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. Nat Neurosci 11: 752-753.
14. Carlini VP, Monzon ME, Varas MM, Cragnolini AB, Schioth HB, et al. (2002) Ghrelin increases anxiety-like behavior and memory retention in rats. Biochem Biophys Res Commun 299: 739-743.
15. Currie PJ, Khelemsky R, Rigsbee EM, Dono LM, Coiro CD, et al. (2012) Ghrelin is an orexigenic peptide and elicits anxiety-like behaviors following administration into discrete regions of the hypothalamus. Behav Brain Res 226: 96-105.
16. Hansson C, Haage D, Taube M, Egecioglu E, Salome N, et al. (2011) Central administration of ghrelin alters emotional responses in rats: behavioural, electrophysiological and molecular evidence. Neuroscience 180: 201-211.
17. Kluge M, Schussler P, Dresler M, Schmidt D, Yassouridis A, et al. (2011) Effects of ghrelin on psychopathology, sleep and secretion of cortisol and growth hormone in patients with major depression. J Psychiatr Res 45: 421-426.

18. Ozsoy S, Besirli A, Abdulrezzak U, Basturk M (2014) Serum ghrelin and leptin levels in patients with depression and the effects of treatment. Psychiatry Investig 11: 167-172.

19. Gonzalez-Quintela A, Alende R, Gude F, Campos J, Rey J, et al. (2008) Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. Clin Exp Immunol 151: 42-50.

20. Stoop JW, Zegers BJ, Sander PC, Ballieux RE (1969) Serum immunoglobulin levels in healthy children and adults. Clin Exp Immunol 4: 101-112.

21. Obiandu C, Okerengwo AA, Dapper DV (2013) Levels of serum immunoglobulins in apparently healthy children and adults in Port Harcourt, Nigeria. Niger J Physiol Sci 28: 23-27.

22. Sakiani S, Olsen NJ, Kovacs WJ (2013) Gonadal steroids and humoral immunity. Nat Rev Endocrinol 9: 56-62.

23. Tennoune N, Legrand R, Ouelaa W, Breton J, Lucas N, et al. (2015) Sex-related effects of nutritional supplementation of Escherichia coli: Relevance to eating disorders. Nutrition 31: 498-507.

24. Goel N, Workman JL, Lee TT, Innala L, Viau V (2014) Sex differences in the HPA axis. Compr Physiol 4: 1121-1155.

25. Leadbeater BJ, Kuperminc GP, Blatt SJ, Hertzog C (1999) A multivariate model of gender differences in adolescents' internalizing and externalizing problems. Dev Psychol 35: 1268-1282.

26. Oldehinkel AJ, Bouma EM (2011) Sensitivity to the depressogenic effect of stress and HPA-axis reactivity in adolescence: a review of gender differences. Neurosci Biobehav Rev 35: 1757-1770.

27. Kryski KR, Smith HJ, Sheikh HI, Singh SM, Hayden EP (2013) HPA axis reactivity in early childhood: Associations with symptoms and moderation by sex. Psychoneuroendocrinology 38: 2327-2336.

28. Marsman R, Swinkels SH, Rosmalen JG, Oldehinkel AJ, Ormel J, et al. (2008) HPA-axis activity and externalizing behavior problems in early adolescents from the general population: the role of comorbidity and gender The TRAILS study. Psychoneuroendocrinology 33: 789-798.

29. Takagi K, Legrand R, Asakawa A, Amitani H, Francois M, et al. (2013) Anti-ghrelin immunoglobulins modulate ghrelin stability and its orexigenic effect in obese mice and humans. Nat Commun 4: 2685.

30. Fetissov SO (2011) Neuropeptide autoantibodies assay. Methods Mol Biol 789: 295-302.

31. Devey ME, Bleasdale-Barr KM, Bird P, Amlot PL (1990) Antibodies of different human IgG subclasses show distinct patterns of affinity maturation after immunization with keyhole limpet haemocyanin. Immunology 70: 168-174.

32. Merbl Y, Zucker-Toledano M, Quintana FJ, Cohen IR (2007) Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. J Clin Invest 117: 712-718.

33. Elkon K, Casali P (2008) Nature and functions of autoantibodies. Nat Clin Pract Rheumatol 4: 491-498.

34. Lutz HU, Binder CJ, Kaveri S (2009) Naturally occurring auto-antibodies in homeostasis and disease. Trends Immunol 30: 43-51.

35. Oldstone MB (2005) Molecular mimicry, microbial infection, and autoimmune disease: evolution of the concept. Curr Top Microbiol Immunol 296: 1-17.

36. Husby G, van de Rijn I, Zabriskie JB, Abdin ZH, Williams RC, Jr. (1976) Antibodies reacting with cytoplasm of subthalamic and caudate nuclei neurons in chorea and acute rheumatic fever. J Exp Med 144: 1094-1110.

37. Bronze MS, Dale JB (1993) Epitopes of streptococcal M proteins that evoke antibodies that cross-react with human brain. J Immunol 151: 2820-2828.

38. Martino D, Giovannoni G (2004) Antibasal ganglia antibodies and their relevance to movement disorders. Curr Opin Neurol 17: 425-432.

39. Swedo SE, Leonard HL, Rapoport JL (2004) The pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS) subgroup: separating fact from fiction. Pediatrics 113: 907-911.

40. Pavone P, Parano E, Rizzo R, Trifiletti RR (2006) Autoimmune neuropsychiatric disorders associated with streptococcal infection: Sydenham chorea, PANDAS, and PANDAS variants. J Child Neurol 21: 727-736.

41. Mell LK, Davis RL, Owens D (2005) Association between streptococcal infection and obsessive-compulsive disorder, Tourette's syndrome, and tic disorder. Pediatrics 116: 56-60.

42. Goines P, Haapanen L, Boyce R, Duncanson P, Braunschweig D, et al. (2011) Autoantibodies to cerebellum in children with autism associate with behavior. Brain Behav Immun 25: 514-523.

43. Singer HS, Morris CM, Gause CD, Gillin PK, Crawford S, et al. (2008) Antibodies against fetal brain in sera of mothers with autistic children. J Neuroimmunol 194: 165-172.

44. Blaser MJ (2014) The microbiome revolution. The Journal of Clinical Investigation 124: 4162-4165.

45. Rhee SH, Pothoulakis C, Mayer EA (2009) Principles and clinical implications of the brain-gut-enteric microbiota axis. Nat Rev Gastroenterol Hepatol 6: 306-314.

46. Mulle JG, Sharp WG, Cubells JF (2013) The gut microbiome: a new frontier in autism research. Curr Psychiatry Rep 15: 337.

47. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, et al. (2015) Altered fecal microbiota composition in patients with major depressive disorder. Brain Behav Immun.

48. Sudo N (2012) Role of microbiome in regulating the HPA axis and its relevance to allergy. Chem Immunol Allergy 98: 163-175.

49. Butters JE (2002) Family stressors and adolescent cannabis use: a pathway to problem use. Journal of Adolescence 25: 645-654.

50. Douglas KR, Chan G, Gelernter J, Arias AJ, Anton RF, et al. (2010) Adverse childhood events as risk factors for substance dependence: Partial mediation by mood and anxiety disorders. Addictive Behaviors 35: 7-13.

51. Ompad DC, Ikeda RM, Shah N, Fuller CM, Bailey S, et al. (2005) Childhood sexual abuse and age at initiation of injection drug use. Am J Public Health 95: 703-709.

52. Sullivan TN, Farrell AD, Kliewer W (2006) Peer victimization in early adolescence: association between physical and relational victimization and drug use, aggression, and delinquent behaviors among urban middle school students. Dev Psychopathol 18: 119-137.

53. Kim KJ, Conger RD, Elder GH, Jr., Lorenz FO (2003) Reciprocal influences between stressful life events and adolescent internalizing and externalizing problems. Child Dev 74: 127-143.

54. King KM, Chassin L (2008) Adolescent stressors, psychopathology, and young adult substance dependence: a prospective study. J Stud Alcohol Drugs 69: 629-638.

55. Pariante CM, Lightman SL (2008) The HPA axis in major depression: classical theories and new developments. Trends Neurosci 31: 464-468.

56. Alink LR, van Ijzendoorn MH, Bakermans-Kranenburg MJ, Mesman J, Juffer F, et al. (2008) Cortisol and externalizing behavior in children and adolescents: mixed meta-analytic evidence for the inverse relation of basal cortisol and cortisol reactivity with externalizing behavior. Dev Psychobiol 50: 427-450.

57. Low NCP, Dugas E, O'Loughlin E, Rodriguez D, Contreras G, et al. (2012) Common stressful life events and difficulties are associated with mental health symptoms and substance use in young adolescents. Bmc Psychiatry 12.

58. Jaffee SR, McFarquhar T, Stevens S, Ouellet-Morin I, Melhuish E, et al. (2015) Interactive effects of early and recent exposure to stressful contexts on cortisol reactivity in middle childhood. J Child Psychol Psychiatry 56: 138-146.

59. Windle M, Wiesner M (2004) Trajectories of marijuana use from adolescence to young adulthood: Predictors and outcomes. Development and Psychopathology 16: 1007-1027.

60. Macleod J, Oakes R, Copello A, Crome I, Egger M, et al. (2004) Psychological and social sequelae of cannabis and other illicit drug use by young people: a systematic review of longitudinal, general population studies. Lancet 363: 1579-1588.

61. Korhonen T, van Leeuwen AP, Reijneveld SA, Ormel J, Verhulst FC, et al. (2010) Externalizing behavior problems and cigarette smoking as predictors of cannabis use: the TRAILS Study. J Am Acad Child Adolesc Psychiatry 49: 61-69.



62. Schisterman EF, Cole SR, Platt RW (2009) Overadjustment bias and unnecessary adjustment in epidemiologic studies. Epidemiology 20: 488-495.

63. van der Pol P, Liebregts N, de Graaf R, Korf DJ, van den Brink W, et al. (2015) Three-Year Course of Cannabis Dependence and Prediction of Persistence. Eur Addict Res 21: 279-290.

64. Huizink AC, Ferdinand RF, Ormel J, Verhulst FC (2006) Hypothalamic-pituitary-adrenal axis activity and early onset of cannabis use. Addiction 101: 1581-1588.

65. van Leeuwen AP, Creemers HE, Greaves-Lord K, Verhulst FC, Ormel J, et al. (2011) Hypothalamic-pituitary-adrenal axis reactivity to social stress and adolescent cannabis use: the TRAILS study. Addiction 106: 1484-1492.

66. Gunnar MR, Wewerka S, Frenn K, Long JD, Griggs C (2009) Developmental changes in hypothalamus-pituitary-adrenal activity over the transition to adolescence: normative changes and associations with puberty. Dev Psychopathol 21: 69-85.

67. Legro RS, Lin HM, Demers LM, Lloyd T (2003) Urinary free cortisol increases in adolescent caucasian females during perimenarche. J Clin Endocrinol Metab 88: 215-219.

68. Romeo RD (2013) The Teenage Brain: The Stress Response and the Adolescent Brain. Curr Dir Psychol Sci 22: 140-145.

69. Colich NL, Kircanski K, Foland-Ross LC, Gotlib IH (2015) HPA-axis reactivity interacts with stage of pubertal development to predict the onset of depression. Psychoneuroendocrinology 55: 94-101.

70. van der Pol P, Liebregts N, de Graaf R, Korf DJ, van den Brink W, et al. (2013) Predicting the transition from frequent cannabis use to cannabis dependence: a three-year prospective study. Drug Alcohol Depend 133: 352-359.

71. van der Pol P, Liebregts N, de Graaf R, Ten Have M, Korf DJ, et al. (2013) Mental health differences between frequent cannabis users with and without dependence and the general population. Addiction 108: 1459-1469.

72. Hoffmann JP, Su SS (1998) Parental substance use disorder, mediating variables and adolescent drug use: a non-recursive model. Addiction 93: 1351-1364.

73. Creemers HE, Korhonen T, Kaprio J, Vollebergh WA, Ormel J, et al. (2009) The role of temperament in the relationship between early onset of tobacco and cannabis use: the TRAILS study. Drug Alcohol Depend 104: 113-118.

74. Marsman R, Oldehinkel AJ, Ormel J, Buitelaar JK (2013) The dopamine receptor D4 gene and familial loading interact with perceived parenting in predicting externalizing behavior problems in early adolescence: the TRacking Adolescents' Individual Lives Survey (TRAILS). Psychiatry Res 209: 66-73.

75. Pagliaccio D, Luby JL, Bogdan R, Agrawal A, Gaffrey MS, et al. (2015) HPA axis genetic variation, pubertal status, and sex interact to predict amygdala and hippocampus responses to negative emotional faces in school-age children. Neuroimage 109: 1-11.

76. Gillespie CF, Phifer J, Bradley B, Ressler KJ (2009) Risk and resilience: genetic and environmental influences on development of the stress response. Depress Anxiety 26: 984-992.

77. Tammimaki AE, Mannisto PT (2010) Are genetic variants of COMT associated with addiction? Pharmacogenet Genomics 20: 717-741.

78. Abdolmaleky HM, Cheng KH, Faraone SV, Wilcox M, Glatt SJ, et al. (2006) Hypomethylation of MB-COMT promoter is a major risk factor for schizophrenia and bipolar disorder. Hum Mol Genet 15: 3132-3145.

79. Xu Q, Ma JZ, Payne TJ, Li MD (2010) Determination of Methylated CpG Sites in the Promoter Region of Catechol-O-Methyltransferase (COMT) and their Involvement in the Etiology of Tobacco Smoking. Front Psychiatry 1: 16.

80. Joubert BR, Haberg SE, Nilsen RM, Wang X, Vollset SE, et al. (2012) 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. Environ Health Perspect 120: 1425-1431.

81. Breitling LP, Yang R, Korn B, Burwinkel B, Brenner H (2011) Tobacco-smoking-related differential DNA methylation: 27K discovery and replication. Am J Hum Genet 88: 450-457.

82. Monick MM, Beach SR, Plume J, Sears R, Gerrard M, et al. (2012) Coordinated changes in AHRR methylation in lymphoblasts and pulmonary macrophages from smokers. Am J Med Genet B Neuropsychiatr Genet 159B: 141-151.

83. Grossniklaus U, Kelly WG, Ferguson-Smith AC, Pembrey M, Lindquist S (2013) Transgenerational epigenetic inheritance: how important is it? Nat Rev Genet 14: 228-235.

84. Li C, Pentz MA, Chou CP (2002) Parental substance use as a modifier of adolescent substance use risk. Addiction 97: 1537-1550.

85. Sasaki M, Kaneuchi M, Sakuragi N, Dahiya R (2003) Multiple promoters of catechol-O-methyltransferase gene are selectively inactivated by CpG hypermethylation in endometrial cancer. Cancer Res 63: 3101-3106.

86. van der Knaap LJ, Riese H, Hudziak JJ, Verbiest MM, Verhulst FC, et al. (2014) Glucocorticoid receptor gene (NR3C1) methylation following stressful events between birth and adolescence. The TRAILS study. Transl Psychiatry 4: e381.

87. Horvath S (2013) DNA methylation age of human tissues and cell types. Genome Biol 14: R115.

88. Johansson A, Enroth S, Gyllensten U (2013) Continuous Aging of the Human DNA Methylome Throughout the Human Lifespan. PLoS One 8: e67378.

89. Koolhaas JM, Bartolomucci A, Buwalda B, de Boer SF, Flugge G, et al. (2011) Stress revisited: a critical evaluation of the stress concept. Neurosci Biobehav Rev 35: 1291-1301.

90. Kirschbaum C, Pirke KM, Hellhammer DH (1993) The 'Trier Social Stress Test'--a tool for investigating psychobiological stress responses in a laboratory setting. Neuropsychobiology 28: 76-81.

91. Gruber AJ, Pope HG, Jr. (2002) Marijuana use among adolescents. Pediatr Clin North Am 49: 389-413.

92. Asbridge M, Duff C, Marsh DC, Erickson PG (2014) Problems with the Identification of 'Problematic' Cannabis Use: Examining the Issues of Frequency, Quantity, and Drug Use Environment. European Addiction Research 20: 254-267.

93. Anaheim B, Rehm J, Gmel G (2008) How to screen for problematic cannabis use in population surveys: an evaluation of the Cannabis Use Disorders Identification Test (CUDIT) in a Swiss sample of adolescents and young adults. European Addiction Research 14: 190-197.

94. Thanki D, Domingo-Salvany A, Barrio Anta G, Sanchez Manez A, Llorens Aleixandre N, et al. (2012) The Choice of Screening Instrument Matters: The Case of Problematic Cannabis Use Screening in Spanish Population of Adolescents. ISRN Addiction 2013: Article ID 723131, 723113 pages.

95. Eekhout I, de Boer RM, Twisk JW, de Vet HC, Heymans MW (2012) Missing data: a systematic review of how they are reported and handled. Epidemiology 23: 729-732.

96. Desai M, Esserman DA, Gammon MD, Terry MB (2011) The use of complete-case and multiple imputation-based analyses in molecular epidemiology studies that assess interaction effects. Epidemiol Perspect Innov 8: 5.

97. Heron J, Hickman M, Macleod J, Munafo MR (2011) Characterizing Patterns of Smoking Initiation in Adolescence: Comparison of Methods for Dealing With Missing Data. Nicotine & Tobacco Research 13: 1266-1275.

98. Ferro MA (2014) Missing data in longitudinal studies: cross-sectional multiple imputation provides similar estimates to full-information maximum likelihood. Ann Epidemiol 24: 75-77.

99. Collins LM, Schafer JL, Kam CM (2001) A comparison of inclusive and restrictive strategies in modern missing data procedures. Psychol Methods 6: 330-351.

100. Eekhout I, Enders CK, Twisk JW, de Boer MR, de Vet HC, et al. (2015) Including auxiliary item information in longitudinal data analyses improved handling missing questionnaire outcome data. J Clin Epidemiol 68: 637-645.

101. Schmidt HD, Shelton RC, Duman RS (2011) Functional biomarkers of depression: diagnosis, treatment, and pathophysiology. Neuropsychopharmacology 36: 2375-2394.

102. Tennoune N, Chan P, Breton J, Legrand R, Chabane YN, et al. (2014) Bacterial ClpB heat-shock protein, an antigen-mimetic of the anorexigenic peptide alpha-MSH, at the origin of eating disorders. Transl Psychiatry 4: e458.

103. Coquerel Q, Sinno MH, Boukhettala N, Coeffier M, Terashi M, et al. (2012) Intestinal inflammation influences alpha-MSH reactive autoantibodies: relevance to food intake and body weight. Psychoneuroendocrinology 37: 94-106.

104. Seiffge-Krenke I, Aunola K, Nurmi JE (2009) Changes in stress perception and coping during adolescence: the role of situational and personal factors. Child Dev 80: 259-279.

# chapter 9

## Summary

The aim of this thesis was to investigate new mechanisms in the relationship between stress and externalizing and internalizing problems in adolescents. Data for this thesis were taken from the TRacking Adolescent´s Individual Lives Survey (TRAILS), a prospective cohort study in which the development of Dutch adolescents from the general population is followed from early adolescence into adulthood.

Studies in animals and in patients with eating disorders or conduct disorder have suggested that antibodies against neuropeptides could play a role in the development of emotional and behavioral problems, and that they might modify the stress response. Therefore, in chapters 2, 3 and 4, we investigated whether autoantibodies (IgG) directed against neuropeptides could influence externalizing and internalizing problems (measured by self-report questionnaires) and hypothalamic-pituitary-adrenal (HPA) axis reactivity (measured during a social stress test) in adolescents. These studies were carried out cross-sectionally at a mean age of 16 years and blood samples from 1230 adolescents were available.

We showed that the levels of autoantibodies against adrenocorticotropic hormone (ACTH) were associated with higher antisocial behavior scores in boys, but with lower antisocial behavior scores in girls (chapter 2). We further demonstrated that in girls, ACTH IgG levels were associated with more internalizing problems, particularly anxiety symptoms (chapter 3). In addition, ACTH IgG levels were associated with a blunted HPA axis reactivity in boys. We also found a weak association of ghrelin-reactive IgM with increased anxiety symptoms in girls (chapter 4). Levels of ghrelin IgG were positively associated with concentrations of influenza IgG (chapter 4). One explanation for this association could be the formation of ghrelin IgG due to molecular mimicry with pathogenic peptides.

These are the first studies on autoantibodies against neuropeptides and relationships with behavioral problems and stress reactivity in the general population, and replication studies are needed to confirm our findings.

In the second part of the thesis the relationship between stress and the development of substance use problems during adolescence was investigated. Patients with a drug addiction often have a history of multiple stressful life events (SLE) and in addition, they often have a changed stress reactivity. However, little is known about the reciprocal relationships between SLE's and substance use as well as whether stress reactivity can predict later problematic substance use. In addition, little is known about modulation of the brain reward system by epigenetic mechanisms and how this could be connected to substance use. Therefore, these questions were addressed in this thesis.

In chapter 5 we demonstrated with the help of a cross-lagged path model that there were reciprocal associations between SLE and cannabis use during adolescence and young adulthood: SLE at mean age 16 predicted cannabis use three years later. In addition, 16 year olds who used cannabis also had a higher chance of experiencing SLE three years later. Cannabis use often co-occurs with increased externalizing problems.

After adjustment for externalizing problems, the associations disappeared. These results show how tightly cannabis use and SLE in adolescence are interconnected, but before these results can lead to any recommendations for new prevention policies, it will be important to determine the exact role of externalizing problems in this relationship.

To test whether stress reactivity can predict future cannabis use problems, the association between the physiological and psychological stress response at age 16 and problematic cannabis use six years later was investigated in a sample of 715 adolescents (chapter 6). Cortisol reactivity during the social stress test was not related to later problematic cannabis use. In cannabis users, high perceived arousal in anticipation of the social stress test was related to a slightly higher risk of developing problematic cannabis use. These results hint towards a role for perceived arousal in predicting problematic cannabis use. Replication is needed to confirm this finding.



Changes in the brain reward system could increase the risk of substance use. Hence, in chapter 7 the relationship between COMT gene methylation and substance use was tested in adolescents with a mean age of 16. We found that *MB-COMT* promoter methylation was related to non-daily smoking, but not with daily smoking or alcohol use. In addition, we tested the interaction between *MB-COMT* promoter methylation and the $Val^{108/158}$Met polymorphism, and showed that adolescents with the Met/Met genotype and high rates of *MB-COMT* promoter methylation were less likely to be frequent cannabis users than adolescents with other genotypes.

This thesis investigated antibodies against neuropeptides, stress and gene methylation as factors in the development of externalizing and internalizing problems. To summarize, our results suggest that there might be a role for neuropeptide autoantibodies in externalizing and internalizing problems as well as a role for ACTH autoantibodies in the regulation of HPA axis reactivity. However, these are the first studies on this subject and more studies are needed to confirm the associations we found. Moreover, the working mechanism of neuropeptide autoantibodies is yet to be determined. Our findings also suggest that it could be interesting for future studies to consider the combined effects of genetic and epigenetic variation when studying the development of substance use. We did not find any relationship between HPA axis reactivity and future problematic cannabis use. However, perceived arousal might have a predictive role for problematic substance use, and this should be investigated further in the future.

## Nederlandse samenvatting

Het doel van dit proefschrift was om nieuwe mechanismen in de relatie tussen stress en externaliserende en internaliserende problemen bij adolescenten te onderzoeken. Voor dit proefschrift zijn gegevens gebruik van de Tracking Adolescent's Individual Lives Survey (TRAILS). TRAILS is een prospectief cohort onderzoek waarin Nederlandse jongeren uit de algemene bevolking vanaf de vroege adolescentie tot in de volwassenheid worden gevolgd in hun ontwikkeling.

Uit dierenonderzoek en bij mensen met eetstoornissen of antisociale gedragsstoornis blijkt dat autoantilichamen tegen neuropeptides en rol kunnen spelen in de ontwikkeling van emotionele en gedragsproblemen, en dat ze misschien invloed hebben op de stress-response. Daarom hebben wij in hoofdstuk 2,3 en 4 onderzocht of autoantilichamen (IgG) gericht tegen neuropeptiden van invloed kunnen zijn op externaliserende en internaliserende problemen (gemeten met vragenlijsten, zelf-rapportage) en hypothalamus-hypofyse-bijnier (HPA) as reactiviteit (gemeten tijdens een sociale stress taak) in adolescenten. Deze cross-sectionele onderzoeken zijn gedaan met gegevens van 1230 adolescenten – gemiddelde leeftijd 16 jaar – van wie de bloedmonsters beschikbaar waren.



Uit de resultaten bleek dat de niveaus van autoantilichamen tegen het adrenocorticotroop hormoon (ACTH) geassocieerd waren met meer antisociaal gedrag bij jongens en met minder antisociaal gedrag bij meisjes (hoofdstuk 2). Daarnaast hadden meisjes met hoge ACTH IgG niveaus in hun bloed meer internaliserende problemen, met name symptomen van angst (hoofdstuk 3). ACTH IgG niveaus waren ook geassocieerd met een lagere HPA as reactiviteit in jongens. We vonden ook een zwakke associatie tussen ghreline-reactieve IgM en verhoogde angstsymptomen bij meisjes (hoofdstuk 4). In dit hoofdstuk beschrijven we verder een verband tussen en hoger niveau van ghreline IgG en hogere concentraties van influenza IgG (hoofdstuk 4). Een verklaring voor deze samenhangen zou een vorming van ghreline IgG als gevolg van 'molecular mimicry' met pathogene peptiden kunnen zijn.

Het tweede deel van het proefschrift gaat over de relatie tussen stress en de ontwikkeling van problemen met middelengebruik tijdens de adolescentie. Het is bekend dat mensen met en verslaving meer stress hebben meegemaakt en vaak en veranderde stress reactiviteit hebben. Maar, er is weinig bekend over de wederzijdse samenhangen tussen stressvolle gebeurtenissen en middelengebruik, en in hoeverre stress-reactiviteit en voorspellende faktor van problematisch middelengebruik is. Ook is er weinig bekend over de modulatie van het beloningssysteem in het hersen door epigenetische mechanismen, en of dit in samenhang staat met middelengebruik. Daarom hebben wij deze vragen verder onderzocht in dit proefschrift.

In hoofdstuk 5 hebben we met hulp van een cross-lagged pad model aangetoond dat stressvolle gebeurtenissen samenhangen met toekomstig cannabis gebruik en vice versa: Het meemaken van stressvolle gebeurtenissen op een gemiddelde leeftijd van 16 jaar voorspelde cannabisgebruik drie jaar later. Echter, een 16-jarige die cannabis gebruikt had ook een grotere kans op het meemaken van meer stressvolle

gebeurtenissen drie jaar later. Vaak gaat cannabisgebruik samen met externaliserend gedrag. Na correctie voor externaliserende problemen verdwenen de verbanden tussen cannabisgebruik en stressvolle gebeurtenissen. Deze resultaten laten zien hoe sterk cannabis gebruik en stressvolle gebeurtenissen in de adolescentie met elkaar zijn verbonden, maar voordat deze resulaten leiden tot concrete preventiemaatregelen, is het belangrijk om eerst nader te onderzoeken wat de exacte rol van externaliserend gedrag is.

Om te onderzoeken of stress reactiviteit en voorspellende waarde heeft voor problematisch cannabis gebruik, hebben we in een steekproef van 715 jongeren getest of de fysiologische (cortisol) en psychologische stress respons bij jongeren van gemiddeld 16 jaar geassocieerd was met problematisch cannabisgebruik 6 jaar later (hoofdstuk 6). Cortisol reactiviteit tijdens de sociale stress taak was niet gerelateerd aan later problematisch cannabisgebruik. Bij cannabisgebruikers vonden wij dat een hoge subjectieve opwinding in afwachting van de sociale stress taak gerelateerd was aan een licht verhoogd risico op het ontwikkelen van problematisch cannabisgebruik. Deze resultaten zouden een aanwijzing kunnen zijn dat subjectieve opwinding een mogelijke rol speelt bij het voorspellen van problematisch cannabisgebruik. Ook hier is replicatie nodig om dit te bevestigen.



Veranderingen in het beloningssysteem van de hersenen kunnen het risico op drugsgebruik verhogen. Daarom hebben wij in hoofdstuk 7 de relatie tussen *COMT*-gen methylering en middelengebruik bij jongeren van gemiddeld 16 jaar onderzocht. We vonden dat *MB-COMT* promotor methylering gerelateerd was aan niet-dagelijks roken, maar er was geen associatie met dagelijks roken en alcoholgebruik. Daarnaast testten we de interactie tussen *MB-COMT* promotor methylering en het $Val^{108/158}Met$ polymorfisme. Hieruit bleek dat adolescenten met het Met/Met genotype en hoge *MB-COMT* promotor methylering minder kans maakten op frequent cannabisgebruik dan adolescenten met andere genotypen.

In dit proefschrift is de samenhang tussen de niveaus van antilichamen, gen methylering, stress en gedrags- en emotionele problemen onderzocht. Samenvattend suggereren de resultaten uit dit proefschrift dat neuropeptide autoantilichamen een rol kunnen spelen in externaliserende en internaliserende problemen en dat ACTH autoantilichamen mogelijk betrokken zijn bij de regulatie van de HPA as. Dit zijn echter de eerste onderzoeken ten aanzien van dit onderwerp en het is belangrijk dat de gevonden verbanden gerepliceerd worden. Bovendien is het van groot belang dat het werkingsmechanisme van neuropeptide autoantilichamen wordt bepaald. Onze bevindingen suggereren verder dat toekomstige studies zowel de genetische als de epigenetische variatie in overweging moeten nemen bij het bestuderen van de ontwikkeling van middelengebruik. Er was geen relatie tussen de HPA-as reactiviteit en toekomstig problematisch cannabisgebruik. Echter, initiale resultaten laten zien dat subjectieve opwinding een voorspellende rol heeft voor problematisch middelengebruik. Ook dit zou in de toekomst verder onderzocht moeten worden.

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## Curriculum Vitae

Johanna Schaefer was born on 22 November 1983 in Berkeley, California. She grew up in Hofheim am Taunus in Germany and graduated from the Friedrich Dessauer Gymnasium in Frankfurt in 2003. She then studied Biochemistry at the Ruhr-Universität Bochum, which she completed in 2006 with a Bachelor thesis focusing on the protective effect of stable dust for the development of allergies in children. She continued to pursue a Research Master in Biomedical Sciences with the specialization ´Immunity and Infection´ at the University of Utrecht, where she investigated EBV immunology and Salmonella virulence factors. After graduating Cum Laude in 2008, she started to work as junior researcher at the department of Biochemistry at the Faculty of Veterinary Medicine at the University of Utrecht on the modulation of host cell lipids by Salmonella. In 2010, she started a Master in international public health at the Vrije Universiteit Amsterdam, in which she focused on the use of geographical information systems for Tuberculosis control in Uganda, where she spent four months during her studies. After graduating in 2011, she briefly worked as a consultant at the Royal Tropical Institute in Amsterdam and taught courses in GIS for public health. She took up a job as junior researcher at the department of Child and Adolescent Psychiatry and Psychology at Erasmus Medical Center in 2012. There, she was involved in a project on the role of neuropeptide autoantibodies for stress reactivity and problem behavior in adolescents. This was a European project in collaboration with the University of Rouen, France, where Johanna worked for six months during her PhD period. In addition to this project, she investigated the relationships between stress and substance use. The combined work on these two projects eventually led to the present PhD thesis. Johanna is currently employed at Excerpta Medica, as associate medical communications manager.

# PhD portfolio

| **PhD candidate:** Johanna Schaefer | **PhD period:** 03/2012 - 10/2015 | |
|---|---|---|
| **Department Erasmus MC:**<br>**Child and Adolescent Psychiatry & Psychology** | **Promotors:**<br>**Prof. dr. Ingmar Franken**<br>**Prof. dr. Frank Verhulst** | |
| | **Supervisor:**<br>**Dr. Floor van Oort** | |
| PhD training | | |
| Courses and workshops | | |
| Course 'Scientific writing' | 2013 | 4 |
| Workshop 'The New Statistics' | 2013 | 0.5 |
| PhD Day (workshops) | 2013 | 0.2 |
| Course 'Research Integrity' | 2013 | 2 |
| Course 'Mixed models' (EpidM, VUmc) | 2014 | 2 |
| Conferences | | |
| LARC Neuroscience meeting Portsmouth/ Poster presentation | 2012 | 1 |
| DOHaD meeting Rotterdam/ Poster presentation | 2012 | 1 |
| ADAA conference San Diego/ Oral presentation | 2013 | 2 |
| ISPNE conference Leiden | 2013 | 1 |
| LARC Neuroscience meeting Rouen/ Oral presentation | 2013 | 2 |
| Teaching | | |
| Supervision MSc thesis (Evangelia Thalassinou, Rotterdam) | 2012-2013 | 1 |
| Supervision laboratory work (Ayako Fukushima, Rouen) | 2012 | 1 |
| Research meetings and symposia | | |
| Research work meetings Rotterdam and Rouen/ presentations | 2012-2014 | 1 |
| TRAILS research meetings Groningen/ oral presentations | 2012-2014 | 1 |
| TRAILS forum Groningen/ oral presentation | 2013 | 1 |
| Parnassia Bavo Minisymposium | 2012 | 0.2 |
| Symposium Stress and Psychopathology (VU) | 2013 | 0.2 |
| UCP symposium Groningen | 2014 | 0.2 |
| Other | | |
| Presentation for grant (Sophia Stichting) | 2012 | 1 |
| Grant proposal writing | 2012 | 1 |

## TRAILS dissertations

Van Hemel-Ruiter, M.E. (2015) Can't take my eyes off of you. The role of cognitive biases, reward sensitivity and executive control in adolescent substance use and abuse. Promotores: Prof.dr. P.J. de Jong, Prof.dr. R.W.H.J. Wiers. Copromotor: Dr. B.D. Ostafin (2 TRAILS articles). University of Groningen.

Van der Knaap, L.J. (2015) Epigenetics and adverse health outcomes. Silenced by the past? Promotores: Prof.dr. F.C. Verhulst, Prof.dr. A.J. Oldehinkel. Copromotores: Dr. F.V.A. van Oort, Dr. H. Riese. Erasmus University Rotterdam.

Booij, S.H. (2015) Dynamics of the human stress system in depression: A combined population- and person-based approach to assess long-term changes and daily life fluctuations. Promotores: Prof.dr. A.J. Oldehinkel, Prof.dr. P. de Jonge. Copromotor: Dr. E.H. Bos (2 TRAILS articles). University of Groningen.

Bennik, E.C. (2015) Every cloud has a colored lining. The relation between positive and negative affect and reactivity to positive and negative events. Promotores: Prof.dr. A.J. Oldehinkel, Prof.dr. J. Ormel. Copromotores: Dr. E. Nederhof, Dr. J.A.C.J. Bastiaansen. University of Groningen.



Rüschoff, B. (2015) Peers in carreers: Peer relationships in the transition from school to work. Promotores: Prof.dr. R. Veenstra, Prof.dr. S.M. Lindenberg. Copromotor: Dr. J.K. Dijkstra. University of Groningen (1 TRAILS article).

Jeronimus, B.F. (2015) Environmental influences on neurotisicm: a story about emotional (in)stability. Promotores: Prof.dr. J. Ormel, Prof.dr. A.J. Oldehinkel. Copromotor: Dr. H. Riese. University of Groningen (TRAILS dissertation in part).

Stavrakakis, N. (2015) Physical activity and depressive symptoms. Is a healthy body necessary for a healthy mind? Promotores: Prof.dr. A.J. Oldehinkel, Prof.dr. P. de Jonge. Copromotor: Dr. A.M. Roest. University of Groningen.

Langenhof, M.R. (2015) Living in a changing world. How early-life development influences animal and human ability to cope with change. Promotores: rof.dr.ir. J. Komdeur, Prof.dr. A.J. Oldehinkel. University of Groningen (TRAILS dissertation in part).

Boelema, S.R. (2014) Alcolhol use in adolescence. A longitudinal study of its effect on cognitive functioning. Promotor: Prof.dr. W.A.M. Vollebergh. Copromotores: Dr. Z. Harakeh, Dr. M.J.E. van Zandvoort. Utrecht University.

Visser, L. (2014) Early detection and prevention of adolescent alcohol use. Parenting and psychosocial factors. Promotor: Prof.dr. S.A. Reijneveld. Copromotor: Dr. A.F. de Winter. University of Groningen.

Mathyssek, C.M. (2014) The development of anxiety symptoms in adolescents. Promotor: Prof.dr. F.C. Verhulst. Copromotor: Dr. F.V.A. van Oort. Erasmus University Rotterdam.

Prince van Leeuwen, A.L. (2013) Blunt vulnerabilities. Identifying risks for initiation and continued use of cannabis in a Dutch adolescent population. Promotores: Prof.dr. A.C. Huizink, Prof.dr. F.C. Verhulst. Copromotor: Dr. H.E. Creemers. Erasmus University Rotterdam.

Laceulle, O.M. (2013) Programming effects of adversity on adolescent adaptive capacity. Promotores: Prof.dr. J. Ormel, Prof.dr. M.A.G. van Aken. Copromotor: Dr. E. Nederhof. University of Groningen.

Vink, N.M. (2013) The role of stress in the etiology of asthma. Promotores: Prof.dr. H.M. Boezen, Prof. dr. J.G.M. Rosmalen, Prof.dr. D.S. Postma. University of Groningen.

Griffith-Lendering, M.F.H. (2013) Cannabis use, cognitive functioning and behaviour problems. Promotores: Prof.dr. H. Swaab. Copromotor: Dr. S.C.J. Huijbregts. University of Leiden.

Marsman, H. (2013) HPA-axis, genes and environmental factors in relation to externalizing behaviors. Promotor: Prof.dr. J.K. Buitelaar. The Radboud University Nijmegen Medical Centre.

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Jaspers, M. (2012) Prediction of psychosocial problems in adolescents : do early childhood findings of the preventive child healthcare help? Promotores: Prof.dr. S.A. Reijneveld, Dr. A.F. de Winter. University of Groningen.

Ivanova, K.O. (2012) From parents to partners : the impact of family on romantic relationships in adolescence and emerging adulthood. Promotores: Prof.dr. M.C. Mills, Prof.dr. R. Veenstra. University of Groningen.

Janssens, K.A.M. (2011) The etiology of functional somatic symptoms in adolescents. A new perspective on lumping and splitting. Promotores: Prof.dr. J.G.M. Rosmalen, Prof.dr. A.J. Oldehinkel. University of Groningen.

Wigman, J.T.W. (2011) Persistance of the extended psychosis phenotype in young people: Link between vulnerability and clinical need. Promotores: Prof.dr. W.A.M. Vollebergh, Prof.dr. J. van Os. University of Utrecht.



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Sijtsema, J.J. (2010) Adolescent aggressive behavior - Status and stimulation goals in relation to the peer context. Promotor: Prof.dr. S. Lindenberg. Co-promotor: Dr. R. Veenstra. University of Groningen.

Buschgens, C.J.M. (2010). It runs in the family – Early biological factors and family environment in children with ADHD symptoms. Promotores: Prof.dr. J.K. Buitelaar, Prof.dr. M.A.G. van Aken. The Radboud University Nijmegen Medical Centre.

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Sondeijker, F.E.P.L. (2006) Neuroendocrine and autonomic risk factors for disruptive behaviors in adolescents. Promotores: Prof.dr. F.C. Verhulst, Prof.dr. J. Ormel. Copromotor: Dr. R.F. Ferdinand. Erasmus University Rotterdam.