SUPPLEMENTARY MATERIAL (Online)

Influence of life stress on depression: Moderation by a polymorphism in the 5-HTT gene, by A. Caspi, K. Sugden, T. E. Moffitt, A. Taylor, I. W. Craig, HL Harrington, J. McClay, J. Mill, J. Martin, A. Braithwaite, R. Poulton.

Materials and Methods

Research sample. Participants were members of the Dunedin Multidisciplinary Health and Development Study. The birth cohort of 1,037 children (52% male) was established at age 3 when the investigators enrolled 91% of the consecutive births between April 1972 and March 1973 in Dunedin, New Zealand. Cohort families represent the full range of socioeconomic status in the general population of New Zealand's South Island. Follow-ups have been carried out at ages 3, 5, 7, 9, 11, 13, 15, 18, 21, and most recently at age 26, when we assessed 96% of the living cohort members. The sample and its history are described in detail elsewhere (S1).

Serotonin transporter genetic variation. We selected to study the 5-HTT gene based on two criteria. (a) Evidence of functionality and (b) evidence that it may moderate response to stress. The promoter activity of the 5-HTT gene, located on 17q11.2, is modified by sequence elements within the proximal 5' regulatory region, designated the serotonin transporter gene-linked polymorphic region (5-HTTLPR). A 20-23 base pair repeat motif within this region occurs as 2 prevalent alleles: One consisting of 14 repeats (the short allele 's') and another of 16 repeats (the long allele 'l'). This polymorphic region has functional significance; 'l/l' homozygote lymphoblast cells produce 1.4-1.7 times the concentration of 5-HTT mRNA than 's/l' and 's/s' cells, uptake of labeled serotonin in 'l/l' homozygote lymphoblast cells is 2 times greater than in 's/l' or 's/s' cells, and the protein produced from 'l/l' cells binds 30-40% more serotonin than cells with the short variant (S2). Although the short promoter variant has

not been conclusively linked to depression, experimental paradigms, including studies of 5-HTT knockout mice (S3), stress-reared macaques (S4), and human functional neuroimaging (S5) have shown that the 5-HTT gene can interact with environmental conditions to shape reactions to stressful experiences, suggesting to us the hypothesis that variations in the 5-HTT gene may explain why stress leads to depression in some people but not in others.

DNA extraction and genotyping. When the Study members were age 26 years, we obtained DNA from 953 participants (97% of those assessed at that age; 51% male); 93% of the DNA samples were obtained via blood and 7% via buccal swabs for those not wishing to undergo phlebotomy. DNA was extracted from blood samples using standard procedures (S6, S7). A modified procedure was used to extract DNA from buccal cells (S8). Primer sequences for 5-HTTLPR are described by Gelernter *et al.* (S9), the forward primer having the sequence (5'- ATGCCAGCACCTAACCCCTAATGT-3') and the reverse (5'-GGACCGCAAGGTGGGCGGGA-3'). This amplifies a 419 base pair product for the 16 repeat ('l') allele and a 375 base pair product for the 14 repeat ('s') allele. PCR was carried out on a PTC-225 DNA engine (MJ Research), using the following cycling conditions: initial 15-min denaturing step at 95°C, followed by 35 cycles of 94°C for 30 sec, 66°C for 30 sec and 72°C for 40 sec, and a final extension phase of 72°C for 15 min. Reactions were performed in 10X reaction Buffer IV (ABgene), 1.5mM $MgCl_2$, 50ng of genomic DNA, 5pmols of each primer, 0.3mM dNTPs and 1 unit of Native *Taq* (Promega). PCR products were separated on a 2.5% agarose gel (MultiABgarose, ABgene) supplemented with Ethidium bromide (0.03%, BDH) and visualised by ultraviolet transillumination.

Population stratification can probably be ruled out as a confounding factor in this study. First, cohort members reporting Maori ethnicity (7%) were not included in our analysis. Second, a genomic control approach based on latent class analysis was adopted, which suggested that the Caucasian sample was genetically homogeneous (S10). Third, allele

frequencies among the non-Maori members of our study were consistent with previously reported allele frequencies in Caucasian populations (S11): 57% for the 16 repeat ('l') allele and 43% for the 14 repeat ('s') allele. No other alleles were detected.

We followed the well-documented functional classification described by Lesch et al. (S2). The sample was split into three groups on the basis of genotype, s/s (N=147, 17% of sample, 51% male), s/l (N=435, 51% of sample, 51% male) and l/l (N=265, 31% of sample, 51% male). The three groups were in Hardy-Weinberg equilibrium ($\chi^2(2)=1.91$, p=0.41), and there was no significant difference in genotype frequencies between the sexes ($\chi^2(2)=0.02$, p=0.99).

Stressful life events were assessed at age 26 with the aid of a life history calendar (S12), a highly-reliable method for ascertaining life-event histories (S13). The 5-year reporting period covered events occurring after the 21st birthday and before the 26th birthday. Events included employment problems (long-term unemployment; being made redundant; losing a job because the company moved; being fired); financial problems (problems with debt, such as having items repossessed; not having enough money to pay for food or household expenses; lacking money for medical expenses; difficulty paying bills); housing problems (homelessness; multiple residential changes); health problems (a disabling physical illness lasting a month or more; a disabling injury); and relationship problems (being involved in a physically violent relationship; a break-up of a cohabiting, intimate relationship). To ensure that the collection of information on life events was not influenced by knowledge of psychiatric outcomes, this information was gathered from Study members by a different interviewer in a separate session. 30% of the Study members experienced no stressful life events, 25% experienced 1 event, 20% 2 events, 11% 3 events, and 15% 4 or more events. Males experienced more stressful life events than females, $X^2(4) = 10.6$, p = .03. There were no significant differences between the three genotype groups in the number of life events they experienced, F (2,846) = .56, *P* = .59,

suggesting that 5-HTTLPR genotype did not influence exposure to stressful life events in adulthood.

Childhood maltreatment. To assess children's experience of stressful life events, we measured their experience of maltreatment between ages 3 to 11 years, as previously described by Caspi et al. (S14). Evidence of childhood maltreatment was ascertained using behavioral observations, parental reports, and retrospective reports by the Study members. First, mother-child interactions were observed during the child's age-3 assessment. The mother was rated by an observer on eight categories: mother's affect toward the child was consistently negative; harshness toward the child; rough, awkward handling of the child; no effort to help child; unaware or unresponsive to child's needs; indifferent to child's performance; demanding of child's attention; soiled, unkempt appearance of child. Mothers engaging in 2 or more such behaviors were classified as rejecting. Second, harsh discipline was measured at ages 7 and 9 using a checklist on which parents indicated if they engaged in ten disciplinary behaviors such as "smack him or hit him with something." Parents scoring in the top decile of the sample-wide distribution were classified as unusually harsh, relative to the culture in which this cohort grew up. Third, changes in the person occupying the role of the child's primary caregiver were ascertained at each assessment. Children who experienced 2 or more such changes during the first decade of life were classified as having suffered disruptive caregiver changes. Fourth, exposure to child physical abuse was assessed retrospectively at age 26 as part of an interview about victimization. Study members were classified as physically abused if they reported multiple episodes of severe physical punishment (e.g., strapping leaving welts; whipping with electric cords) resulting in lasting bruising or injury before age 11. Fifth, unwanted sexual contact was assessed retrospectively at age 26 as part of an interview about reproductive health. Study members were classified as sexually abused if they reported having their genitals touched, touching another's genitals, or attempted/ completed sexual intercourse before age 11.

We derived a cumulative exposure index for each child by counting the number of maltreatment experiences during the first decade of life; in the full sample, 64% of the children experienced no maltreatment, 27% experienced 1 indicator of maltreatment, and 9% experienced 2 or more indicators of maltreatment. There was no significant association between the three genotype groups and maltreatment ($X^2(4) = 1.67$, $p = .80$), suggesting that 5-HTTLPR genotype did not influence exposure to maltreatment in childhood.

Depression outcomes at age 26. Depression was assessed at age 26 using the Diagnostic Interview Schedule (S15), administered by clinicians with a medical or clinical psychology degree. The reporting period was 12 months prior to interview, which occurred within 60 days of the 26th birthday. This structured interview yields a continuous measure of depressive symptoms (M = 5.2, SD = 10.5; Cronbach's alpha = .95) as well as a diagnosis of a major depressive episode according to DSM-IV criteria (S16). The essential feature of a major depressive episode is a period of at least two weeks during which there is either depressed mood or the loss of interest or pleasure in all activities. One must also experience four of the following additional symptoms: changes in weight or appetite, sleep, or psychomotor activity; decreased energy; feelings of worthlessness or guilt; difficulty thinking or concentrating; or recurrent thoughts of death or suicidal ideation. Lastly, the episode must be accompanied by clinically significant distress or impairment in social, occupational or other important areas of functioning. 17% of Study members (58% female vs. 42% male; OR = 1.6, 95% CI: 1.1-2.2) met criteria for a past-year major depressive episode, which is comparable to age and sex prevalence rates observed in U.S. epidemiological studies (S17). In addition to analyzing the diagnostic outcome of depression, we also examined specific evidence of suicide ideation/attempt; 3% of the Study members reported suicide attempts or recurrent thoughts about suicide in the context of a depressive episode. We also collected informant reports about

symptoms of depression for 96% of Study members at age 26, by mailing a brief questionnaire to persons nominated by each Study member as "someone who knows you well." Informants were best friends, partners, or other family members. Using a 3-point scale (0 = no, doesn't apply; 1 = applies somewhat; 2 = certainly applies), informants rated the Study member on 4 different symptoms: "feels depressed, miserable, sad, or unhappy," "feels that no one loves them," "seems lonely," and "talks about suicide" (M = 1.0; SD = 1.2; Cronbach's alpha = .80).

Measures of depression at ages 18 and 21. Depression symptoms and diagnoses were derived in the same way at ages 18 and 21 as at age 26 (described above). Study members were interviewed with the Diagnostic Interview Schedule at ages 18 and 21 years (S18). At those assessments, the interviews covered the 12-month periods prior to the 18th (age 17 years) and 21st (age 20 years) birthdays.

Statistical analysis. We used a moderated regression framework (S19) to estimate the association between depression and (a) 5-HTTLPR genotype, (b) stressful life events, and (c) their interaction. Sex was entered into the regressions as a covariate. The equation for the models is as follows

Depression = b0 + b1(Sex) + b2(5-HTTLPR) + b3(Stress) + b4(5-HTTLPR * Stress),

where,

b0 is the intercept,

b1 is the regression coefficient associated with the effect of sex, which is coded as:

0 = female; 1 = male,

b2 is the regression coefficient associated with the effect of variations in the serotonin transporter gene promoter, which is here coded to reflect the number of long ('l') alleles, such that:

0 = ss; 1 = sl; 2 = ll,

b3 is the coefficient associated with the effect of stressful life events, coded to reflect the number of life events, such that:

0 = no stressful events;
1 = 1 stressful event;
2 = 2 stressful events;
3 = 3 stressful events;
4 = 4+ stressful events,

b4 is the coefficient associated with the interaction effect, which is the product of the two variables (5-HTTLPR and Stressful Life Events). For continuous measures (self-reports and informant reports of depression symptoms), we used ordinary least squares (OLS) regression; for categorical measures (diagnosis of major depression and suicide ideation/attempt), we used logistic regression.

The full results of these regression analyses are provided in Supporting Tables S1 through S3. The coefficients (labeled as b) in all the Supporting Tables are the model parameters for each type of model (e.g., OLS, logistic) before any transformation (e.g., exponentiation to obtains odds ratios). Predicted values can be plotted using variable values.

In additional analyses we examined the moderating effect of 5-HTTLPR on the association between stress and depression, as a function of MAOA genotype. Genotyping details about MAOA are provided in Caspi et al. (S14). Study members were grouped as "low" MAOA activity (carrying the 2, 3 or 5 repeat variants; 61% male) and "high" MAOA activity (carrying the 3.5 or 4 repeat variants; 75% male). As the gene is situated on the X chromosome, only females are heterozygous (23% of the sample). We observed that the influence of life stress on depression was moderated by variation in the 5-HTT gene, regardless of individuals' MAOA genotype. Among carriers of an 's' allele, the effect of stressful life events on depression was consistently significant, whether they had low- or high-MAOA activity status (Supporting Table S4). In contrast, among l/l homozygotes, the effect of

stressful life events on depression was nonsignificant, regardless of MAOA status (Supporting Table S5).

Assessing the robustness of the G x E effect. We incorporated five analytic features into this study to test the robustness of the G x E effect. First, we tested that the G x E interaction on depression obtained whether stress occurred in childhood or in adulthood. Second, we tested that the G x E interaction predicted within-individual increases in depression from a baseline measured before life events occurred. Third, we tested that the G x E interaction was not an artefact of genetic vulnerability evoking life events. Fourth, we used informant reports of depression to rule out the possibility of self-report biases. Fifth, we examined multiple outcome measures, which is of particular importance in the behavioral sciences because different measurements have different sources of error associated with them (S20). Conducting multiple tests is problematic in the following situation: when (a) several tests are conducted, (b) only a small subset of the tests attain significance, and (c) the small number that attain significance can be explained by chance. This situation is even more problematic if (d) no hypothesis was stated in advance, or (e) researchers selectively report only the test that attained significance. In contrast, as in the present study, multiple statistical tests can provide evidence that a finding is robust in the following situation: (a) several tests are conducted using different methods of measurement and analysis, (b) all findings are in the same direction and all of the tests attain significance (or very near-significance), and (c) this number of significant tests exceeds the proportion that could be explained by chance. This situation provides even better evidence of a sturdy finding if, as in the present study, (d) a clear hypothesis was stated in advance, and (e) the researchers collect multiple outcome measures and report all of them to document that the finding is not an artefact of one measurement approach.

SUPPORTING REFERENCES AND NOTES

S1. T. E. Moffitt, A. Caspi, M. Rutter, P. A. Silva, *Sex Differences in Antisocial Behavior: Conduct Disorder, Delinquency, and Violence in the Dunedin Longitudinal Study* (Cambridge Univ. Press, Cambridge, 2001).

S2. K. P. Lesch *et al., Science* **274**, 1527 (1996).

S3. D. L. Murphy *et al., Brain Res. Bull*. **56**, 487 (2001).

S4. A. J. Bennett *et al., Mol. Psychiatry* **7**, 188 (2002).

S5. A. R. Hariri et al., *Science* **297**, 400 (2002).

S6. D. Bowtell, *Anal. Biochem.* **162**, 463 (1987).

S7. M. Jeanpierre, *Nucleic. Acids. Res*. **15**, 9611 (1987).

S8. B. Freeman *et al*., *Behav. Genet.* **33,** 67 (2002).

S9. J. Gelernter, H. Kranzler, J. F. Cubells, *Hum. Genet*. **101**, 243 (1997).

S10. G. A. Satten, D. Flanders, Q. Yang, *Am. J. Hum. Genet.* **68** 466 (2001).

S11. K. P. Lesch, in *Behavioral Genetics in the Postgenomics Era*, R. Plomin, J. C. DeFries, I. W. Craig, P. McGuffin, Eds. (APA, Washington, DC, 2003), pp. 389-424.

S12. A. Caspi *et al., Int. J. Methods Psychiatric Res*. **6**, 101 (1996).

S13. R. F. Belli, W. L. Shay, F. P. Stafford, *Public Opin. Quart*. **65**, 45 (2001).

S14. A. Caspi *et al., Science* **297**, 851 (2002).

S15. L. N. Robins, L. Cottler, K. Bucholtz, W. Compton, *Diagnostic Interview Schedule for DSM-IV* (Washington University, St. Louis, MO, 1995).

S16. *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, Washington, DC, ed. 4, 1994).

S17. R. C. Kessler, K. A., McGonagle, M. Swartz, D. G. Blazer, C. B. Nelson, *J. Affect. Disorders* **29**, 85 (1993).

S18. S. R. Jaffee *et al*. *Arch. Gen. Psychiatry* **58** 215 (2002).

S19. L. S. Aiken, S. G. West, *Multiple Regression: Testing and Interpreting Interactions* (Sage, Thousand Oaks, CA, 1991).

S20. Institute of Medicine, *Reducing risks for mental disorders* (Washingon, DC, National Academy Press, 1994).

Table S 1. Results of final regression analyses testing G x E interaction effects on indices of depression at age 26. *The Table presents final models with main effects and interactions entered simultaneously*. For continuous measures (self-reports and informant reports of depression symptoms), we used ordinary least squares (OLS) regression; for categorical measures (diagnosis of major depressive episode [MDE] and suicide ideation/attempt), we used logistic regression (see Statistical Analysis section for details).

Depression outcomes at age 26	**Predictor Variables**															
	Intercept	**Sex**				**5-HTTLPR**				**Life events, ages 21-26**				**5-HTTLPR x Life events**			
		b	**se**	**t/z**	**p**	**b**	**se**	**t/z**	**p**	**b**	**se**	**t/z**	**p**	**b**	**se**	**t/z**	**p**
Self-report of depressive symptoms	3.32	-2.44	0.69	3.53	0.001	0.49	0.75	0.65	0.52	2.57	0.48	5.39	0.001	-0.89	0.37	2.39	0.02
Increase in self-report of depressive symptoms*	3.69	-1.12	0.67	1.67	0.100	0.44	0.72	0.62	0.54	1.80	0.47	3.84	0.001	-0.71	0.36	1.97	0.05
Diagnosis of MDE	-2.29	-0.62	0.20	3.11	0.002	0.25	0.24	1.03	0.30	0.56	0.13	4.35	0.001	-0.19	0.10	1.91	0.056
First diagnosis of MDE†	-2.93	-0.84	0.27	3.08	0.002	0.53	0.33	1.61	0.11	0.77	0.19	4.11	0.001	-0.34	0.15	2.37	0.02
Suicide ideation/attempt	-5.42	-0.07	0.38	0.17	0.870	0.98	0.58	1.71	0.09	0.91	0.28	3.22	0.001	-0.39	0.20	1.95	0.05
Informant report of depressive symptoms	0.61	-0.22	0.08	2.73	0.006	0.12	0.09	1.38	0.17	0.32	0.06	5.84	0.001	-0.11	0.04	2.54	0.01

* This regression equation contains an additional covariate which controls for self-reports of depression symptoms collected during diagnostic interviews with the Study members at ages 18 and 21 years. The model thus tests whether the 5-HTTLPR x Life events interaction predicts within-individual increases in depression symptoms over time.

†This regression equation excludes from analysis Study members who met diagnostic criteria for depression prior to age 21 (27%). The model thus tests whether the 5-HTTLPR x Life events interaction predicts new cases of depression at age 26 years.

Table S2. Results of final regression analyses testing G x E interaction effects on depressive symptoms at age 26 years, and on depressive symptoms at the age-21 and age-18 assessments. *The Table presents final models with main effects and interactions entered simultaneously.* The G x E interaction predicts depression occurring after life events (row 1), but not depression that occurred before life events (rows 2 and 3).

Self-reports of depression symptoms	**Predictor Variables**																
	Intercept	**Sex**				**5-HTTLPR**				**Life events, ages 21-26**				**5HTTLPR x Life events**			
		b	**se**	**t**	**p**	**b**	**se**	**t**	**p**	**b**	**se**	**t**	**p**	**b**	**se**	**t**	**p**
Depression symptoms, age 26	3.32	-2.44	0.69	3.53	0.001	0.49	0.75	0.65	0.52	2.57	0.48	5.39	0.001	-0.89	0.37	2.39	0.02
Depression symptoms, age 21	6.39	-2.69	0.76	3.53	0.001	-0.07	0.83	-0.09	0.93	2.18	0.53	4.09	0.001	-0.53	0.41	1.29	0.20
Depression symptoms, age 18	5.56	-4.20	0.70	6.01	0.001	0.30	0.76	0.40	0.69	1.67	0.49	3.40	0.001	-0.17	0.38	0.44	0.66

Table S3. Results of final regression analyses testing G x E interaction effects on indices of depression. *The Table presents final models with main effects and interactions entered simultaneously.* The first row shows the analysis predicting diagnosis of major depressive episode (MDE) at age 26 years; for this analysis, we used logistic regression. The second row shows a supplementary analysis, predicting the number of depression episodes experienced by Study members (range 0-3, as assessed according to independent psychiatric interviews carried out when the Study members were aged 18, 21, and 26 years old); for this analysis, we used a negative binomial regression. Childhood stressful events was treated as a single quantitative variable in the regression analyses, ranging from no maltreatment (= 0), to probable maltreatment (= 1), to severe maltreatment (=2).

Depression outcomes	**Predictor Variables**															
	Intercept	**Sex**				**5HTT**				**Childhood stressful events, ages 3-11**				**5HTT x Childhood events**			
		b	**se**	**z**	**p**	**b**	**se**	**z**	**p**	**b**	**se**	**z**	**p**	**b**	**se**	**z**	**p**
Any MDE, (ages 18-26)	-0.39	-0.91	0.15	6.02	0.001	0.02	0.13	0.12	0.90	0.70	0.21	3.27	0.001	-0.33	0.16	2.01	0.05
Number of age periods with MDE diagnosis (ages 18, 21, 26)	-0.72	-0.54	0.11	5.01	0.001	0.05	0.10	0.51	0.61	0.51	0.13	3.87	0.001	-0.22	0.10	2.10	0.04

Table S4. The association between stressful life experiences and depression among individuals with either one or two copies of the 5-HTTLPR 's' allele, as a function of MAOA genotype. We used logistic regression analyses to examine the association between young-adult stress and major depression episode at age 26 years, and negative binomial regression analyses to examine the association between childhood stress and number of adult depression episodes between ages 18-26. Sex was a covariate in analyses carried out among the low- and high-MAOA activity groups, but not among the intermediate-MAOA activity group, as the MAOA gene is situated on the X chromosome and only females are heterozygous.

Individuals with a 5-HTTLPR 's' allele												
	MAOA genotype											
	Low-MAOA activity genotype (n = 141)				**Intermediate-MAOA activity genotype (n = 134)**				**High-MAOA activity genotype (n = 300)**			
	b	SE	z	p	b	SE	z	p	b	SE	z	p
Young-adult stress —> depression at age 26	.67	.17	3.98	.001	.43	.17	2.56	.01	.33	.11	3.00	.003
Childhood stress —> adult depression	.40	.16	2.57	.01	.33	.18	1.84	.07	.40	.12	3.25	.001

Table S5. The association between stressful life experiences and depression among individuals homozygous for the 5-HTTLPR 'l' allele, as a function of MAOA genotype. See Table S4 for details.

Individuals homozygous for the 5-HTTLPR 'l' allele												
	MAOA genotype											
	Low-MAOA activity genotype (n = 62)				**Intermediate-MAOA activity genotype (n = 57)**				**High-MAOA activity genotype (n = 140)**			
	b	SE	z	p	b	SE	z	p	b	SE	z	p
Young-adult stress —> depression at age 26	-.03	.32	.10	.92	.21	.24	.87	.39	.17	.21	.84	.40
Childhood stress —> adult depression	.06	.30	.21	.84	.02	.27	.07	.94	-.08	.27	.32	.75