N-methyl-D-Aspartate receptors (NMDAR) are ionotropic glutamate receptors that are involved in the regulation of nearly every process in the brain. Therefore, even a subtle disturbance in NMDAR function may result in severe pathological consequences. Loss-of-function mutations in the NMDAR-encoding genes have been implicated in numerous neuropsychiatric disorders, including intellectual disability, developmental delay, schizophrenia, autism spectrum disorders, epilepsy, and movement disorders. Insufficient NMDAR function can be rectified by positive allosteric modulators, including neurosteroids; however, the mechanism underlying the potentiating effect of steroids is not well understood.

By employing patch-clamp electrophysiology we assessed the effect of newly synthesized neurosteroid-like pregnane analogues on recombinant GluN1/GluN2B receptors. We demonstrated that compounds with short C3 residues, such as pregnanolone acetate (PA-Ace) and pregnanolone carboxylate (PA-Car), are negative modulators of NMDAR, whereas compounds with longer C3 residues, such as pregnanolone butyrate (PA-But) and epipregnanolone butyrate (EPA-But), are positive modulators of NMDARs. Furthermore, we revealed that EPA-But has a disuse-dependent positive allosteric effect, being similar in that regard to endogenous neurosteroid pregnenolone sulfate (PE-S).

Combining electrophysiology, molecular biology, and computational modelling, we identified the PE-S and EPA-But binding sites at the transmembrane domain of the GluN1/GluN2B receptor. Our results indicate that EPA-But binds the NMDAR at the GluN1(M4)/GluN2B(M1), GluN2B(M4)/GluN1(M1), and GluN2B(M1/M4) interfaces. In contrast, PE-S binds the receptor only at the GluN2B(M1/M4) interface. Moreover, we proposed the mechanisms by which the steroids potentiate NMDAR function.

Next, we characterized the effect of ten *de novo* disease-associated mutations in the hGluN2B subunit on the receptor functional properties and surface expression. In addition, we evaluated the effect of EPA-But and PE-S at NMDARs harbouring disease-associated mutations in hGluN1 and hGluN2B subunits. Our results uncovered the potential of EPA-But and PE-S in compensation for the effect of loss-of-function mutations.

In this study, we revealed structural principles underlying the potentiating effect of steroids. Our results open up new possibilities for developing new steroid-based drugs for treating disorders associated with the hypofunction of NMDAR.