ZFPM1 necessary for development of serotonergic projections related to anxiety and contextual fear learning

Journal Club review of: Inactivation of the GATA cofactorZFPM1 results in abnormal development of dorsal raphe serotonergic neu-ron subtypes and increased anxiety-like behaviour. Tikker, L., Casarotto, P., Singh, P., Biojone, C., Piepponen, P., Estartus, N., Seelbach, A., Sridharan, R., Laukkanen, L., Castren, E., and Partanen, J. (2020). The Journal of Neuroscience: the official journal of the Society for Neuroscience, 40(45):JN–RM–2252–19–8682.

Authors: Masakazu Taira[1] & Miles J Desforges[1]

[1] Neural Computation Unit, Graduate School of Okinawa Institute of Science and Technology Graduate University, Okinawa 904-0495, Japan

Corresponding authors: masakazu.taira@oist.jp, or miles.desforges@oist.jp.

Neural Computation Unit, Okinawa Institute of Science and Technology Graduate University, Okinawa 904-0495, Japan. +81-(0)98-966-8824

The serotonergic system is strongly implicated in anxiety and depression, and it is the first-line target for pharmacological treatment (Tamblyn et al., 2019). Genetic factors predispose people to these disorders, but the anatomical and molecular diversity of the main serotonergic nuclei, the dorsal raphe (DR) and median raphe (MR), and their extensive projections make establishing causal links between genes, physiology, and etiology complex. Nevertheless, genome-wide screening can highlight correlations with genetic or epigenetic markers (Li et al., 2016), whose effects can then be tested through gene knockout in animal models.

A genome-wide screening by Baselmans et al. (2015) found that *Zfpm1* methylation was negatively correlated with factors associated with well-being. ZFPM1 is a zinc-finger cofactor that interacts with the GATA family of transcription factors and is expressed in certain serotonergic and GABAergic neurons from early development to adulthood. Therefore, Tikker et al. (2020) recently used selective gene knockout to examine the role of ZFPM1 in development and behaviour.

Tikker et al. (2020) generated *Zfpm1* conditional knockout (*Zfpm1*^{CKO}) mice in which *Zfpm1* was knocked out selectively in rhombomere 1, an embryonic hindbrain segment in which serotonin neurons are derived. The number of serotonergic neurons was reduced in the ventrolateral DR (DRVL) and increased in the dorsal DR (DRD) in *Zfpm1*^{CKO} mice. Notably, a substantial number of DRD serotonergic neurons expressed the DRVL-specific marker BCL11B, and there was decrease in the number of serotonergic cells expressing this marker in DRVL. No change was detected in ventral DR (DRV) or B8 MR areas, and importantly, the total number of neurons across DR nuclei was unaffected. Based on these findings, the authors hypothesized that *Zfpm1* knockout disrupted cell positioning, rather than generation. The authors speculate that *Zfpm1* regulates positioning by repressing the transcriptional processes that cause medial migration of serotonergic precursors, and thus knocking out *Zfpm1* causes DRVL neurons to be displaced to DRD.

Notable effects of *Zfpm1* knockout were also produced at DR projection sites. The density of serotonergic axons was greatly reduced in the dorsal hippocampus (dHPC), ventral hippocampus (vHPC), dorsal lateral geniculate nucleus, and superior colliculus. In contrast the density increased in the basolateral amygdala (BLA), although less consistently than the reductions seen elsewhere.

Knockout of *Zfpm1* also affected mouse behaviour. In an elevated plus maze, *Zfpm1*^{CKO} mice made less open arm entries than control littermates, indicating anxiety-like behaviour. In a recognition task, *Zfpm1*^{CKO} mice spent less time than controls exploring novel objects. *Zfpm1*^{CKO} mice also showed more freezing during contextual fear conditioning compared with controls, and this effect was abolished by treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine. Tikker et al. (2020) tested for depression-like behaviours with a forced swim test but found no significant differences between control and *Zfpm1*$^{\mathrm{CKO}}$ mice, and both groups responded equally positively to fluoxetine in this test. Overall, these results suggest that alterations in specific projections led to enhanced fear and anxiety-like responses in *Zfpm1*^{CKO} mice, while behaviours dependent on spared serotonergic projections were unaffected.

The two key findings of Tikker et al. (2020) were that *Zfpm1* is critically involved in normal development of the DR serotonergic system and that alterations in *Zfpm1* produce anxiety-like and fear-related behaviours. The paper also raised two important questions. Firstly, how do the serotonergic axonal density changes produced by *Zfpm1* knockout align with our current understanding of DRVL and DRD projections? Secondly, how does each of the affected areas contribute to the behavioural phenotype seen?

The reduction in hippocampal serotonergic afferents in *Zfpm1*^{CKO} mice is surprising if attributed solely to the displacement of DRVL to DRD. In a previous tracing study,

Muzerelle et al. (2016) explicitly noted a lack of projections from the DRVL to the hippocampus. This suggests *Zfpm1* knockout either affected a hippocampal-projecting area not examined by Tikker et al. (2020) or altered axonal targeting of neurones in the examined DR regions without affecting cell placement. Serotonergic projections to the hippocampus include dense efferents from the caudal DR (DRC) (B6), MR (B8), and some sparse efferents from DRV and DRD (Soiza-Reilly and Gaspar, 2020). Of these, only the DRC was not specifically examined by Tikker et al. (2020). It may be that *Zfpm1* knockout affected the DRC, which is adjacent to the DRD, but this was missed because these neurons were included with other regions during analysis. Substantiating this possibility, it was noted the DRVL region in which significant change was found was situated caudally. Alternatively, Tikker et al. (2020) may have limited their analysis to B7 cells, which includes the DRD, DRVL and DRV but excludes DRC, and so did not note changes in the DRC. Complicating the DRC hypothesis, Kast et al. (2017) found the majority of DRC serotonergic neurons project strongly to the vHPC but not the dHPC, whereas Tikker et al. (2020) found both hippocampal regions were equally affected. Still another possibility is that *Zfpm1* knockout altered axonal targeting in the hippocampal projecting MR without affecting cell location. A further experiment using retrograde tracers in the hippocampus of $Zfpm1^{CKO}$ mice might shed light on the curious decrease in hippocampal serotonergic axon density.

The enhanced anxiety-related behaviour and contextual fear conditioning reported by Tikker et al. (2020) are consistent with the known functions of the regions affected by *Zfpm1* knockout. In particular, the DRVL is strongly linked to anxiety-like behaviours. The intrinsic excitability properties of DRVL neurons appears to mediate greater activation by stressful stimuli (Crawford et al., 2010). For example, one previous study showed that an elevated T-maze task increased c-fos expression specifically in the DRVL (Vilela-Costa et al., 2019).

That same study also showed that chronic, but not acute, fluoxetine treatment increased time spent in the open arm, decreased c-fos expression in non-serotonergic cells, and increased c-fos expression in serotonergic cells. 5-HT1A heteroreceptors on non-serotonergic cells were speculated as a mechanism for this shift. The effect of fluoxetine suggests DRVL serotonergic output is anxiolytic, while DRVL non-serotonergic activity may be anxiogenic. Consequently, anxiety-like behaviour may arise in *Zfpm1*^{CKO} mice through both decreased serotonergic output in DRVL projection sites and increased non-serotonergic activity in the DRVL.

Reduction in the number of serotonergic neurons in DRVL was mirrored by an increase in DRD. This increase may also be instrumental in the anxiety-like behavioural phenotype. Ren et al. (2018) found that activation of DR projections to the central amygdala (CeA) promoted anxiety-like behaviours. In addition, activity of serotonergic neurons in the CeA strongly increased after foot shock (Ren et al., 2018). And inhibition of CeA neurons that express 5-HT2A receptors downregulates learned fear responses (Isosaka et al., 2015). Accordingly, increased DRD serotonin output in the CeA in response to foot shock may contribute to the enhanced contextual fear conditioning.

As the hippocampus is associated with contextual fear learning and anxiety-like behaviours, reduced serotonergic afferents could play a key role. In contextual fear conditioning, hippocampal neurons have been proposed to represent the contextual cues that come to elicit a conditioned response by exciting the BLA, which in turn activates the CeA to produce fear responses (Maren et al., 2013). Waider et al. (2019) showed that knockout of *Tph2*, which is necessary for serotonin production, increased activity in the dHPC along with fear responses. Conversely, Mlinar and Corradetti (2018) found that application of serotonin in the dHPC decreased activity. Therefore, *Zfpm1* knockout may enhance fear responses by

reducing serotonergic inhibition in the dHPC, thus enhancing activation of BLA. Notably, *Zfpm1* knockout also slightly increased the number of serotonergic axons in the BLA itself. Furthermore, the BLA has been linked to neophobia (Sarowar et al., 2017), another behavioural phenotype reported by Tikker et al. (2020).

Intriguingly, chronic serotonin depletion in the hippocampus, as presumably occurred in *Zfpm1*^{CKO} mice, appears to paradoxically produce anxiogenic effects similar to those of acute serotonin activation. For example, Ohmura et al. (2020) found optogenetic activation of serotonergic terminals in vHPC was anxiogenic. This seeming contradiction may mirror the effect of SSRIs in acutely increasing synaptic serotonin, worsening anxiety and depression, but chronically re-balancing release and uptake to alleviate symptoms. One long-term effect of serotonin in the hippocampus which may contribute to improving symptoms is upregulation of neurogenesis. Mahar et al. (2014) describe how chronic unpredictable stress decreases both DR output and hippocampal neurogenesis, producing depression-like behaviour, all of which are alleviated by chronic SSRIs. Tikker et al. (2020) performed a test for depression, the forced swim test, and they found fluoxetine-induced immobility reduction was unaffected by *Zfpm1*^{CKO} mice. However, Mahar et al. (2014) discuss how fluoxetine decreases immobility independently of hippocampal neurogenesis. Therefore, the reduction in serotonergic afferents to the hippocampus in *Zfpm1*^{CKO} mice may have reduced neurogenesis without influencing the forced swim test. Further investigation of depression-like behaviour and hippocampal neurogenesis may be fruitful in *Zfpm1*^{CKO} mice.

As a final point of interest, the role of *Zfpm1* in hippocampal function may not be limited to the developmental effects discussed here. Li et al. (2016) identified *Zfpm1* as a novel gene that is methylated in the hippocampus following acute stressful restraint. This suggests a dynamic role for *Zfpm1* in the adult brain, which warrants future examination.

In conclusion, Tikker et al. (2020) found that *Zfpm1* is required for the normal development of DRVL serotonergic neurons that project to brain regions involved in anxiety and fear learning. The behavioural phenotype found in *Zfpm1*^{CKO} mice suggests *Zfpm1* may be a useful genetic or epigenetic etiological marker for investigating the heritability of anxiety-related disorders.

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